Tissue damage and inflammation induced by snake venoms

Bruno Lomonte

Department of Clinical Immunology, University of Göteborg, Göteborg, Sweden

and

Instituto Clodomiro Picado, Faculty of Microbiology, University of Costa Rica, San José, Costa Rica

Göteborg, Sweden
1994

TISSUE DAMAGE AND INFLAMMATION INDUCED BY SNAKE VENOMS

Bruno Lomonte
Department of Clinical Immunology, University of Göteborg, S-413 46 Göteborg, Sweden, and Instituto Clodomiro Picado, Faculty of Microbiology, University of Costa Rica, San José, Costa Rica

ABSTRACT

Some characteristics of the local tissue damage and inflammatory reactions induced by snake venoms were analyzed in a mouse model. Tissue damage was studied by intravital, light, and electron microscopic techniques, and by the use of biochemical markers. Detailed information on the early development and dynamics of local tissue damage was obtained by intravital microscopy. Main alterations were microvascular plasma leakage, hemorrhage, blood flow disturbances, thrombosis, and myonecrosis. A new technique for the quantification of myonecrosis *in vivo* was established, based on the principle of MTT reduction. The method was tested for its usefulness in the evaluation of antibody-mediated neutralization of myotoxicity.

The inflammatory response to venom included early lymphopenia and neutrophilia, thrombocytopenia, with edema and leukocyte extravasation at the site of injection. A rapid plasma peak of IL-6 was induced by venoms, as well as by purified muscle-damaging and hemorrhagic toxins, in response to cellular damage.

The effects of a purified hemorrhagic toxin on cultured endothelial cells were studied. The toxin was not directly lytic to these cells even at high concentrations, and caused moderate cell detachment due to its metalloproteinase activity, suggesting that the endothelial cell damage *in vivo* occurs via an indirect mechanism, probably initiated by proteolytic degradation of the basal lamina of microvessels.

Myotoxin II from *Bothrops asper* venom was shown to lyse a variety of cell types in culture, including myoblasts and endothelial cells. This property was exploited in a cytotoxicity assay for the evaluation of myotoxin neutralization. Heparin was found to be a potent inhibitor of its cytolytic activity, by forming complexes, held at least in part by electrostatic interactions. The ability of heparin to neutralize several related non neurotoxic phospholipase A_2 myotoxins present in crotalid venoms, was not dependent on its anticoagulant effect. Thus, non-anticoagulant heparin has a potential as a therapeutic aid, which should be further evaluated. The structural characteristic of the binding interaction of heparin with myotoxin II were analyzed, indicating that a hexasaccharide is the minimal heparin chain size required, and that both N-sulfate and O-sulfate groups participate in the binding.

ISBN 91-628-1162-2

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I Lomonte, B., Tarkowski, A. and Hanson, L.Å. (1993) Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* **17**, 93-105.

II Calderón, L., Lomonte, B., Gutiérrez, J.M., Tarkowski, A. and Hanson, L.Å. (1993) Biological and biochemical activities of *Vipera berus* (European viper) snake venom. *Toxicon* **31**, 743-753.

III Lomonte, B., Gutiérrez, J.M., Romero, M., Núñez, J., Tarkowski, A. and Hanson, L.Å. (1993) An MTT-based method for the *in vivo* quantification of myotoxic activity of snake venoms and its neutralization by antibodies. *Journal of Immunological Methods* **161**, 231-237.

IV Lomonte, B., Lundgren, J., Johansson, B. and Bagge, U. (1994) The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. *Toxicon* **32**, 41-55.

V Lomonte, B., Gutiérrez, J.M., Borkow, G., Ovadia, M., Tarkowski, A. and Hanson, L.Å. (1994) Activity of hemorrhagic metalloproteinase BaH-1 and myotoxin II from *Bothrops asper* snake venom on capillary endothelial cells *in vitro*. *Toxicon* (in press).

VI Lomonte B., Tarkowski, A., Bagge, U. and Hanson, L.Å. (1994) Neutralization of the cytolytic and myotoxic activities of phospholipases A_2 from *Bothrops asper* snake venom by glycosaminoglycans of the heparin/heparan sulfate family. *Biochemical Pharmacology* (in press).

VII Lomonte, B.and Maccarana, M. (1994) The interaction between heparins and myotoxin II, a Lys-49 phospholipase A_2 from *Bothrops asper* snake venom: relationship of binding to neutralization of cytotoxic action (manuscript).

TISSUE DAMAGE AND INFLAMMATION INDUCED BY SNAKE VENOMS

Dissertation for the degree of Doctor of Philosophy to be publicly examined at the University of Göteborg, in the lecture hall, 3rd floor, Microbiology building, Guldhedsgatan 10, Göteborg, on April 8, 1994, at 9:00 a.m.

by

Bruno Lomonte
MQC, MSc

"To observe without thinking is as dangerous as to think without observing."

From *La Aventura del Trabajo Intelectual*, by A. Zubizarreta.

To Margarita and Irene.

CONTENTS

LIST OF ABBREVIATIONS

a.a. : amino acid
ATP : adenosine triphosphate
CHO : Chinese hamster ovary cells
CK : creatine kinase
ED_{50} : effective dose 50%
EDTA : ethylenediaminetetraacetic acid
EIA : enzyme-immunoassay
EM : electron microscopy
FCS : fetal calf serum
FITC : fluorescein isothiocyanate
GAG : glycosaminoglycan
HPLC : high performance liquid chromatography
i.v. : intravenous
i.m. : intramuscular
i.d. : intradermal
IFN : interferon
IL : interleukin
kD : kilodalton
L6 : skeletal muscle myoblast cells
LA : low affinity
LD_{50} : lethal dose 50%
LDH : lactic dehydrogenase
LPS : lipopolysaccharide
LT : leukotriene
MAb : monoclonal antibody
MED : minimal edema-forming dose
MHD : minimal hemorrhagic dose
M_r : molecular weight
mRNA : messenger ribonucleic acid
MTT : 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide
PAF : platelet-activating factor
PAGE : polyacrylamide gel electrophoresis
PBS : phosphate-buffered saline
PG : prostaglandin
PLA_2 : phospholipase A_2
s.c. : subcutaneous
SDS : sodium dodecylsulfate
TBS : Tris-buffered saline
tEnd : capillary endothelial cells
TGF-β : transforming growth factor-beta
TNF-α : tumor necrosis factor-alpha

INTRODUCTION

Medical importance of venomous snakes

Human envenomations due to snakebites are a significant health problem, especially in tropical regions of the world. As these regions mostly correspond to underdeveloped nations, and since snakebites occur largely in remote rural areas, epidemiological data are generally scarce and most likely underestimate the true situation. Nevertheless, some available figures and estimations of incidence and mortality reported for several countries might illustrate the magnitude of this problem.

The World Health Organization, analyzed in 1954 statistical data on deaths caused by snakebites on a global basis (Swaroop and Grab, 1954). In this classical report, an estimation of 30,000-40,000 deaths per year was obtained, with the highest figures corresponding to Asia (25,000-30,000) and Latin America (3,000-4,000). The global incidence of snakebites was estimated as 500,000 per year. More recent reports indicate that snakebite mortality is still high in some areas of the world. Warrell and Fenner (1993) estimated 20,000 annual deaths from snakebites in India, and 50,000-100,000 annual deaths in the world. In Burma (Myanmar), 2,000 annual deaths were reported in 1930 (15.4/100,000 inhabitants), while still 1,000 annual deaths (3.3/100,000) were registered for 1980, currently constituting the fifth cause of death in this nation (Lwin *et al*., 1985; Warrell, 1993). Cardoso *et al*. (1993) described data from Brazil on 19,200 snakebites per year with 25% mortality in the pre-antivenom era (original reports dated between 1911 and 1918), 23,470 cases with 2.3% mortality in 1943, and 20,170 cases with 0.6% mortality in 1986-1989. In the tribe of Waorani indians of Eastern Ecuador, it was estimated that 4% of all deaths were due to snakebites (Larrick *et al*., 1978). Pugh and Theakston (1980) reported a snakebite incidence of 497/100,000 with 12.2% mortality, and an estimated 10,000 annual deaths, in savanna Nigeria.

In addition to being a cause of mortality, snakebites may lead to permanent disability of the victim in a proportion of cases, due to the severe tissue damage caused by the venom of some species. This point is further considered in the following sections.

The relevance of venomous snakes as a cause of human morbidity and mortality has been pointed out above. In addition, snake venoms are medically important from a basic point of view. Biologically-active components present in these secretions, selected through millions of years of evolutionary pressures (Davidson and Dennis, 1990), enclose a great potential as tools for the study and understanding of their

pharmacological targets. For example, snake neurotoxins have been invaluable in the study of voltage-regulated ion channels, neurotransmitter release, and nicotinic acetylcholine receptor structure and function (Castle *et al*., 1989; Whittaker, 1990). Cobra venom factor has been an important tool for the study of the complement system and its role in a variety of pathological conditions (Harrison and Lachmann, 1986; Vogt, 1990). *Bothrops jararaca* venom was the tool that led to the discovery of bradykinin (Rocha e Silva *et al*., 1949). Many snake venom enzymes and peptides are exploited in the field of coagulation research (Stocker, 1990*a*; Kornalík, 1991), diagnosis (Stocker, 1990*b*), and clinical applications (Furukawa and Ishimaru, 1990). Undoubtedly, many interesting agents and actions still await to be discovered in the toxic secretions of snakes (Dufton, 1993).

Venomous snakes and their venoms

About 15% of the approximately 2500-3000 known species of snakes are venomous, and they can be taxonomically classified in five families: Viperidae, Crotalidae, Elapidae, Hydrophiidae, and Colubridae (Mengden, 1983; Mehrtens, 1987). Of these, Viperidae, Crotalidae, and Elapidae are of major medical importance (Warrell and Fenner, 1993).

Snake venoms are produced by a pair of specialized exocrine glands in the mouth (Kochva *et al*., 1980) and are complex mixtures of molecules of different chemical nature (Jiménez-Porras, 1970; Tu, 1977). For example, using sensitive analytical separation techniques such as capillary electrophoresis, as many as 70 peptides can be detected in a single venom (Perkins *et al*., 1993). The best characterized venom components include proteins (with or without enzymatic activities) and peptides, which display a wide variety of pharmacological and toxic actions. Historically, venoms from a given family have been classified according to their main pharmacological activity, such as "neurotoxic", "hemotoxic", "hemorrhagic", and so on. However, these categorizations are too simplistic, and seem misleading in the view of present knowledge on the complexity of venom actions and their heterogeneity among different species of a given family (Russell *et al*., 1975). As pointed out by Warrell (1993) when referring to these classifications, "a more critical attitude towards species diagnosis and the development of specific, sensitive and practicable immunodiagnostic techniques has exposed many exceptions to this simple scheme, which should now be abandoned".

The different activities of snake venoms can usually be attributed to particular components or toxins, although it is possible that different toxins may act

synergistically in inducing an effect, and that a given toxin may have more than one activity (Jiménez-Porras, 1968, 1970; Kini and Iwanaga, 1986).

Local tissue damage induced by snake venoms

Venoms from many species of snakes are able to cause, in addition to major systemic toxic effects, striking alterations at the site of injection, leading to a variable degree of local tissue damage (Ohsaka, 1979). The local effects of snake venoms have been classically defined as hemorrhage, myonecrosis, and edema (Tu and Homma, 1970; Ohsaka, 1979). The magnitude of these effects depends on the venom type, the route and dose injected, and the host. In severe cases, the tissue damage may lead to serious sequelae such as permanent tissue loss, disability or amputation (Rosenfeld, 1971; Ho *et al*., 1986*b*; Kerrigan, 1991; Nishioka and Silveira, 1992).

The recognition of the clinical importance of local tissue damage in snakebites has motivated an increasing number of studies on its pathogenesis. It may be assumed that a better understanding of the mechanisms of tissue damage will result in improved therapeutic modalities. In addition, the action of toxins may reveal mechanisms of cell and tissue injury that are common to other pathologic conditions.

In early studies, crude venoms were utilized. However, soon it became evident that, in order to dissect the complex events leading to the development of tissue lesions, the respective components had to be isolated and studied individually. Efforts to isolate specific toxins responsible for the tissue-damaging activities of venoms have resulted in the discovery of a number of proteins, some of which have been extensively characterized at the structural and biochemical level. Nevertheless, the relatively large number of studies dealing with the isolation and biochemical characterization of venom components, does not parallel studies of their *in vivo* pathogenic actions and mechanisms. For example, more than 50 different snake toxins capable of inducing hemorrhage have been isolated and biochemically characterized, but only few of them have been studied regarding the pathogenesis of their hemorrhagic action in experimental models (Ownby, 1990).

It should be pointed out that, although the study of isolated toxins constitutes an invaluable approach to dissect and analyze venom actions in detail, the effects induced by whole, unfractionated venoms should always be considered as an important reference frame. As stated above, particular venom actions might be the result of synergistic effects of two or more components. As well, the induction of a given effect by a particular isolated component is not necessarily informative of its relative contribution or importance in the context of the crude venom actions.

Like other pathological conditions, local tissue damage induced by venoms can be studied by two basic types of approaches. One is to analyze material obtained from human cases of snakebite, such as tissue biopsies and biological fluids, together with the clinical signs and symptoms. This approach has many inherent limitations, as it is mainly descriptive, with experimental manipulations being difficult to control. However, it is most valuable, as it directly examines the effects of venoms in the human. As an alternative, experimental animal models are utilized with the assumption that the effects of venoms will be similar to those observed in man. Although some examples of species differences in the susceptibility to tissue-damaging toxins have been reported, they seem to be uncommon, and in general, similar venom effects are seen in clinical cases and in animals commonly used for research. Harris and coworkers have described that notexin, a potent pre-synaptic neurotoxin and myotoxin from the venom of *Notechis scutatus scutatus*, causes much more extensive muscle damage in the rat than in the mouse (Harris, 1991). Chang and Tseng (1978) described that crotamine, a myotoxin from the venom of *Crotalus durissus terrificus*, while having a potent action on mouse and rat muscle, did not affect muscles from frog and chicken. Another exception might be the case of several *Micrurus* spp. venoms, which induce muscle damage in mice (Gutiérrez *et al*., 1983; 1986*b*; 1993), although no clinical reports of muscle damage have yet appeared (Harris and Cullen, 1990). Nevertheless, the possibility that some degree of muscle damage has gone undetected in patients bitten by these species has not been ruled out. Intense myalgia has been recently reported in patients envenomed by *Micrurus corallinus* and *M. frontalis* (Coelho *et al*., 1992).

In addition to experimental animal models, cell cultures represent potentially useful tools for the study of tissue damaging toxins, and have increasingly been utilized in recent years (Hayes and Bieber, 1986; Hodges *et al*., 1987; Ziolkowski and Bieber, 1992; Takacs *et al*., 1992; Brusés *et al*., 1993; Baker *et al*., 1993; Bultrón *et al*., 1993*b*; and present work). The obvious advantage of these *in vitro* cellular systems is the possibility to control variables in a more defined fashion. However, the results cannot always be easily extrapolated to the *in vivo* situation, as cultivable cell lines are often less differentiated than normal, fully mature tissues (for example skeletal muscle myoblasts vs. mature muscle), or may correspond to anatomical sites different from the tissues of interest (for example umbilical cord endothelial cells vs. capillary endothelium in specific organs and tissues).

A number of snake toxins with the ability to induce local tissue damage have been isolated. Although the knowledge on their structural and functional aspects is

progressing, their precise mechanisms of action are still largely unknown. This is in contrast with the case of snake toxins which act upon the nervous system, the knowledge of their target structures and mechanisms of action being more advanced.

Tissue-damaging toxins isolated from snake venoms

The following section summarizes the current knowledge on the main three types of local tissue-damaging venom components: hemorrhagic toxins, myotoxins, and edema-inducing toxins. The experimental techniques commonly utilized for their study are also shortly mentioned.

a. Hemorrhagic toxins

Many toxins capable of inducing hemorrhage at the site of injection or at distant sites have been purified from the venoms of crotalids and viperids (Bjarnason and Fox, 1988; Ownby, 1990). All these toxins are proteins and display variable degrees of proteolytic activity *in vitro*, on general substrates such as casein or dimethylcasein. The biochemical characterization of these enzymes has proven them to be zinc metalloproteinases (Bjarnason and Fox, 1988). Few hemorrhagic toxins have been sequenced to date (either directly or by their cDNA), although the number of available sequences is rapidly increasing. The comparison of these sequences indicates that, despite differences in molecular weight, all of them appear to be related through a common ancestral gene, and do not bear similarity to any other known protein, except for their zinc binding site (Hite *et al*., 1992*a*; Paine *et al*., 1992). Very recently, however, a significant homology has been observed with a group of reproductive tract proteins involved in the maturation of sperm and in sperm-egg fusion (Gomis-Rüth *et al*., 1993; Bjarnason and Fox, 1993). On the basis of molecular weight, hemorrhagic toxins can be classified into three groups, the "small" metalloproteinases (~25 kD, 200-205 amino acid residues), the "medium" (30-60 kD), and the "large" (60-90 kD), the latter having a more potent *in vivo* activity (Bjarnason and Fox, 1988; Paine *et al*., 1992; Kini and Evans, 1992).

A recently recognized structural feature of hemorrhagic toxins is the presence of a disintegrin domain in the group of "large" toxins (Hite *et al*., 1992*a*; Paine *et al*., 1992). Disintegrins are low molecular weight (5-9 kDa), cystein-rich peptides, found in the venom of many snake species, which all contain an Arg-Gly-Asp (RGD) sequence. This conserved sequence allows them to potently inhibit platelet aggregation, and is now known to be the site of interaction with cellular integrins (therefore the name disintegrins) (Gould *et al*., 1990; Scarborough *et al*., 1993). Thus,

hemorrhagic toxins share a common precursor with disintegrin peptides (Hite *et al*., 1992*a*; Paine *et al*., 1992; Kini and Evans, 1992) and can actually release disintegrins by autoproteolysis (Takeya *et al*., 1993) . Since the "large" hemorrhagic toxins are also the most biologically active, it has been speculatively hypothesized that the disintegrin domain may function as a specific targeting unit for endothelium, enhancing the efficiency of these metalloproteinases (Takeya *et al*., 1990). The recent molecular cloning of several hemorrhagic toxins (Hite *et al*., 1992*b*; Bjarnason and Fox, 1993; Moura-da-Silva *et al*., 1993) will allow to construct and express the appropriate mutants to test this hypothesis.

During the last two decades, a proliferation in the number of purified hemorrhagic toxins from snake venoms occurred. The relationship between their proteolytic activity and the mechanism of hemorrhage was disputed during some time, mainly due to two factors. On one hand, some of the toxins were initially described as not being proteolytic. This apparent discrepancy was subsequently found to be caused by the lack of sensitive substrates and by suboptimal assay conditions, and it is now well established that all snake hemorrhagic toxins are metalloproteinases. On the other hand, a lack of correlation between the *in vivo* hemorrhagic potency and the *in vitro* proteolytic activity was observed, when using general substrates (i.e., Mandelbaum *et al*., 1984). However, further work demonstrated a better correlation between enzymatic and biological activities of the toxins, by using more specific substrates, such as glomerular basal lamina preparations or purified basal lamina components (Ohsaka *et al*., 1973; Bjarnason *et al*., 1988). The demonstration of the ability of purified hemorrhagic toxins to proteolytically degrade several major extracellular matrix components *in vitro*, such as collagen type IV, fibronectin, entactin/nidogen, and laminin (Baramova *et al*., 1989, 1990*a*, 1991; Bjarnason *et al*., 1988; Shannon *et al*., 1989; Maruyama *et al*., 1992), has led to the proposal of a mechanism of action based on the enzymatic digestion of basal lamina of the microvasculature (Ohsaka *et al*., 1973; Bjarnason and Fox, 1988; Bjarnason *et al*., 1988). Methods for the measurement of the proteolytic degradation of extracellular matrix produced by endothelial cells in culture have been reported (Takacs *et al*., 1992). *In vivo* studies analyzing the supramolecular organization of the extracellular matrix constituents (Timpl, 1989; Yurchenco and Schittny, 1990) of capillaries, using sensitive immunohistochemical and electron microscopic methods, should shed more light on the precise mechanism of action. However, no such analyses have yet been performed with hemorrhagic toxins applied *in vivo*.

Several histopathological studies of the microvessel damage caused by purified hemorrhagic toxins have been reported. By electron microscopic analysis of the affected tissues, it has been confirmed that the basal lamina of capillaries is often disrupted (Ownby *et al*., 1978, 1990; Ownby and Geren, 1987; Moreira *et al*., 1994). However, another major *in vivo* finding is an extremely rapid effect on endothelial cells, which undergo degenerative morphological changes that can progress until complete ruptures or gaps are formed within the cells (Ownby *et al*., 1978; 1990; Ownby and Geren, 1987; Moreira *et al*., 1994). The observation of this phenomenon suggests the possibility that hemorrhagic toxins could have a direct action on endothelial cells, as has been theoretically discussed (Ownby and Geren, 1987; Ownby *et al*., 1990; Gutiérrez and Lomonte, 1989). Alternatively, endothelial cell alterations might be an indirect consequence of the proteolytic degradation of their extracellular matrix environment. Studies that evaluate the direct actions of purified hemorrhagic toxins on cultured endothelial cells are lacking. A recent report presenting evidence for the induction of apoptosis of endothelial cells in culture, as a possible basis for the hemorrhagic action of venoms, is difficult to interpret, as it was performed using crude venoms (Araki *et al*., 1993). Furthermore, apoptosis is an active cellular process, often requiring gene expression (Sen, 1992; Williams and Smith, 1993; Collins and Lopez, 1993), and thus, relatively long time, in comparison to the speed by which endothelial cell damage and hemorrhage develop *in vivo*.

Indeed, any proposed mechanism of action for the hemorrhagic toxins should consider the fact that microvessels lose their integrity *in vivo* within the range of a few minutes (Ownby *et al*., 1990; and present work). In this regard, it is of interest to point out that the degradation of basal lamina constituents by hemorrhagic toxins *in vitro* seems a relatively slow process: proteolytic degradation of the substrates is often demonstrated using incubation times of 4 to 24 hr (Bjarnason *et al*., 1988; Shannon *et al*., 1989; Baramova *et al*., 1990*a*, 1991). While this does not deny the role of proteolysis in the hemorrhagic effect, its implications for the mechanism of action have not been widely discussed in the literature. It might be that only partial or minor cleavages on certain key constituents of the basal lamina are necessary to destabilize the structural integrity of vessels. Another possibility, although entirely speculative, could be that a few initial specific cleavages activate in turn an endogenous mechanism, that would rapidly amplify the degradation of the basal lamina. Finally, it is also possible that the proteolytic activity of hemorrhagic toxins on intact basal lamina constituents *in vivo* would be higher than under artificial, *in vitro* conditions. In conclusion, although the currently available evidence clearly points out at a

hemorrhagic mechanism based on the proteolytic attack of vascular basal lamina, the details of the process are still incomplete, especially *in vivo*.

Concerning the mode of erythrocyte extravasation induced by these toxins, two alternatives exist, and evidence has been presented for both types of mechanisms, apparently depending on the particular toxin. One alternative is hemorrhage *per diapedesis*, in which red blood cells leave the vessels through the widened intercellular junctions of endothelial cells. The other is hemorrhage *per rhexis*, in which erythrocytes escape via true transcellular discontinuities or gaps formed within the damaged endothelial cells, without an opening of their intercellular junctions (Ownby, 1990). A hemorrhagic toxin purified from the venom of *Trimeresurus flavoviridis* was shown to act *per diapedesis* (Oshio, 1984). On the other hand, several hemorrhagic toxins isolated from the venoms of *Crotalus atrox*, *C. horridus horridus*, *Agkistrodon bilineatus*, and *Bothrops asper* have been shown to induce erythrocyte extravasation *per rhexis* (Ownby *et al*., 1978; 1990; Moreira *et al*., 1994).

Several methods have been developed for the quantification of the biological potency of hemorrhagic toxins (reviewed by Bjarnason and Fox, 1988). The most utilized is the skin assay of Kondo *et al*. (1960), which involves the intradermal injection of varying amounts of the test substance in rabbits or mice, followed by a measurement of the area of the hemorrhagic spot, viewed from the internal side of the dissected skin. The potency (or activity) is expressed as the "Minimal Hemorrhagic Dose" (MHD), which is arbitrarily defined as the amount of toxin that induces a hemorrhagic spot of 10 mm in diameter, in a time of 2-24 hr, depending on different researchers. The MHD values of different hemorrhagic toxins range from 0.04 to 11 µg (Ownby, 1990). An alternative method is based on the quantification of hemoglobin in extracts of muscular tissue after injection of the tested agent (Ownby *et al*., 1984*a*).

b. Myotoxins

Toxins that induce degeneration of skeletal muscle cells are also widely distributed in snake venoms (Mebs *et al*., 1983). From a biological point of view, this should not be surprising, as skeletal muscle comprises about 80% of the body mass of mammals (Harris and Cullen, 1990), and since snake venoms are functionally important not only for the killing of the prey, but also for its digestion (Dufton, 1993). Muscle-damaging toxins have been classified into two to four groups, according to different researchers: Ownby (1990) and Mebs and Ownby (1990) divided them into two main groups, (1) small, basic, non-enzymatic myotoxins, and (2) phospholipase

A_2 myotoxins. Harris and Cullen (1990) included the same two groups, and an additional group formed by (3) the cardiotoxins. Gutiérrez and Cerdas (1984) added still a fourth group, including (4) hemorrhagic toxins that may damage skeletal muscle fibers indirectly, perhaps through ischemia. These four main types of muscle-damaging proteins, and their subgroups, are listed in Table 1.

Table 1: A classification of muscle-damaging toxins from snake venoms.

Group	Characteristics
(1) Small myotoxins	Basic, non-enzymatic, single chain peptides of 42-45 a.a.
(2) Cardiotoxins	Basic, non-enzymatic, single chain proteins of ~60 a.a.
(3) PLA_2 myotoxins	
a. neurotoxic	Basic, single chain (~120 a.a.) or complexed proteins with PLA_2 activity and presynaptic neurotoxicity.
b. non-neurotoxic	Basic, single chain (~120 a.a.) or dimeric proteins with PLA_2 structure.
b'. enzymatically-active	Asp-49 PLA_2s
b''. enzymatically-inactive	Lys-49 (or other variant) PLA_2s
(4) Indirect myotoxic factors	May damage skeletal muscle by indirect mechanisms, perhaps through ischemia

(1) Small myotoxins:

The group of "small" myotoxins is formed by highly basic proteins composed of 42-45 amino acid residues, without any known enzymatic activity (Ownby, 1990; Harris and Cullen, 1990). The basic character is a constant feature not only of the small myotoxins, but also of almost all myotoxic proteins isolated to date (Table 1), suggesting that positively charged amino acid residues on the toxins play a central role in the mechanism of cell recognition and/or cell damage. Small myotoxins have

been isolated from several rattlesnake (*Crotalus* spp.) venoms, in which they appear to be widely distributed, as shown by immunological detection (Bober *et al*., 1988).

Two well characterized examples of this homologous family of peptides are crotamine, from *Crotalus durissus terrificus* (Laure, 1975) and myotoxin *a*, from *C. viridis viridis* (Ownby *et al*., 1976; Cameron and Tu, 1977). When injected into mice, these small myotoxins cause immediate prostration, contracture of the hind limbs, and respiratory distress. The earliest histopathological feature of skeletal muscle exposed to these toxins is vacuolization, progressing to necrosis by 48 hr after injection. By electron microscopy, it has been shown that this vacuolation is due to dilatation of the sarcoplasmic reticulum and perinuclear space. Other cells in the tissue such as fibroblasts and endothelial cells are not affected (Ownby *et al*., 1976; Ownby, 1990; Mebs and Ownby, 1990). An interesting feature of small myotoxins is that they are not cytolytic to muscle cell precursors such as myoblasts *in vitro* (Baker *et al*., 1993; Brusés *et al*., 1993), despite their potent myotoxic action *in vivo*.

The molecular mechanism of action of these toxins is not completely established. Both crotamine and myotoxin *a* have been shown to act on a Na^+ channel, increasing the membrane permeability to this ion, on rat and mouse skeletal muscle preparations (Chang and Tseng, 1978; Hong and Chang, 1985). This action would allow the influx of Na^+ and water, which in turn would cause swelling of the endoplasmic reticulum, and eventually, necrosis of the muscle cell (Mebs and Ownby, 1990). Other researchers have proposed that these myotoxins act directly on the sarcoplasmic reticulum, based on evidence for the *in vitro* interaction with the sarcoplasmic reticulum Ca^{2+}-ATPase (Volpe *et al*., 1986; Utaisinchareon *et al*., 1991; Baker *et al*., 1991), and on the cytochemical detection of sarcoplasmic reticulum-bound myotoxin *a* when applied onto muscle sections *in vitro* (Tu and Morita, 1983). However, no evidence for toxin internalization in intact muscle fibers has yet been presented in favour of this hypothesis.

Using a theoretical approach based on sequence comparisons of small myotoxins and other natural myotoxic components, Kini and Iwanaga (1986) have speculatively predicted that the site responsible for myotoxicity corresponds to the cationic region at residues 2-10, which shows the charge pattern +00+++00+, and is followed by a hydrophobic region. However, *in vitro* and *in vivo* studies using synthetic peptides and chemical cleavage fragments of myotoxin *a* suggest that both the amino- and the carboxy-terminal regions of this protein are implicated in the muscle-damaging action (Baker *et al*., 1991).

(2) Cardiotoxins:

Cardiotoxins (also named cytotoxins, membrane toxins, and direct lytic factors) are a group of basic proteins of 60-62 amino acid residues, devoid of enzymatic activity, and structurally homologous to the post-synaptic neurotoxins, or α-neurotoxins (Dufton and Hider, 1991). These membrane-active proteins have been found in the venoms of elapid snakes, including the cobras (genus *Naja*) and ringhals (genus *Hemachatus*). The sequences of about 60 cardiotoxins have been determined. Their three-dimensional structure is basically similar to that of α-neurotoxins, despite the marked difference in their biological activities (Dufton and Hider, 1991; Rees and Bilwes, 1993). The name cardiotoxin originates from the ability of these components to cause cardiac arrest *in vitro* and *in vivo* (Harris and Cullen, 1990; Harvey, 1990). However, they also act as general cytolytic agents towards many cell types, and thus, are also referred to as cytotoxins (Kini and Evans, 1989*a*; Dufton and Hider, 1991). Few studies on the myotoxic activity of cardiotoxins are available. Mebs (1986) reported that cardiotoxins from *Naja nivea* and *N. nigricollis* lacked myotoxicity. However, other studies, using cardiotoxins from the venoms of *Dendroaspis jamesoni* (Duchen *et al*., 1974), *Naja mossambica mossambica* (d'Albis *et al*., 1988) and *Naja naja atra* (Ownby *et al*., 1993), clearly indicate that skeletal muscle damage develops at the site of injection. Microscopic examination of tissue after injection of cardiotoxin-1 from *N. n. atra* revealed prominent myonecrosis, with rupture of the plasma membrane in the area of wedge-shaped lesions (delta lesions), and condensation of myofibrils into dense clumps, alternating with clear areas containing some elements of the sarcotubular system and damaged mitochondria (Ownby *et al*., 1993). The basal lamina of muscle cells was observed to remain intact during the whole degenerative process, and capillary endothelial cells were also spared from damage (Ownby *et al*., 1993). *In vitro*, membrane depolarization followed by visible cell damage of cultured myotubes from chick embryos was obtained after exposure to seven highly purified cardiotoxins (Hodges *et al*., 1987).

The molecular basis for the cytolytic action of cardiotoxins has been studied intensively, but the nature of their membrane receptor or binding site has remained elusive (Harvey, 1990; Dufton and Hider, 1991; Rees and Bilwes, 1993).

(3) Phospholipase A_2 myotoxins:

Phospholipase A_2 (PLA_2; EC 3.1.1.4) myotoxins have been found in the venoms of many snakes of the families Elapidae, Crotalidae and Viperidae (Rosenberg, 1990). As indicated in Table 1, they can be divided into two main

groups: (a) presynaptically neurotoxic and highly lethal myotoxic PLA_2s, and (b) non-neurotoxic, myotoxic PLA_2s with low lethal activity (Mebs and Ownby, 1990).

(3a) presynaptically neurotoxic PLA_2 myotoxins:

Some well characterized toxins of this group are notexin (Halpert and Eaker, 1975; Harris *et al*., 1975), taipoxin (Fohlman *et al*., 1976; Harris and Maltin, 1982), and crotoxin (Fraenkel-Conrat *et al*., 1980; Gopalakrishnakone *et al*., 1984; Kouyoumdjian *et al*., 1986). Besides having a potent presynaptic action (Cull-Candy *et al*., 1976), which accounts for their high lethal activities (mouse i.v. LD_{50} values of 0.017, 0.002, and 0.025 μg/g for notexin, taipoxin, and crotoxin, respectively; Harris, 1991), these toxins induce prominent damage to skeletal muscle. Nevertheless, it is interesting to note that presynaptically-acting PLA_2s are not always myotoxic, one example of this being β-bungarotoxin (Mebs and Ownby, 1990).

Notexin, a single chain PLA_2 from *Notechis scutatus scutatus*, is probably the best studied among this group of toxins, regarding its effects on muscle (Harris *et al*., 1975, 1980; Harris, 1991; Harris and Johnson, 1978; Pluskal *et al*., 1978; Ng and Howard, 1980; Helmke and Howard, 1986; Sharp *et al*., 1993; Hodgson *et al*., 1993). It has a potent myotoxic action: an injection of 2 μg will completely necrose the soleus muscle of a 150 g rat (Harris and Cullen, 1990). The morphological features of muscle damage induced by both groups of PLA_2s (neurotoxic and non-neurotoxic) are generally similar (Harris and Cullen, 1990; Mebs and Ownby, 1990). Degeneration of muscle fibers proceeds rapidly, and is evident by 0.5-3 hr. The earliest alterations are hypercontraction of sarcomeres and the appearance of wedge-shaped membrane lesions (delta lesions). Mitochondria also show signs of damage, such as swelling, abnormal cristae, floccular degeneration, and disruption. Creatine kinase (CK) and other enzymes are rapidly released from the damaged cells, and thus elevations are detectable in blood plasma. The degenerative process is also accompanied by an early edema (but not hemorrhage) and infiltration by phagocytic cells. By 12-24 hr muscle fibers are totally destroyed, with a more amorphous and hyaline appearance. The basal lamina is preserved throughout this process, as well as the microvasculature. Immature satellite cells, situated between the basal lamina and the plasma membrane of muscle fibers, are also left undamaged (Harris and Cullen, 1990; Mebs and Ownby, 1990). This is relevant since satellite cells are believed to be responsible for the formation of myoblasts, myotubes, and the eventual regeneration of skeletal muscle after necrosis, if conditions are adequate.

In similarity with the small non-enzymatic myotoxins described above, the neurotoxic PLA_2 myotoxins are not cytolytic to immature muscle growing in culture (Harris *et al*., 1980; Jiang *et al*., 1987; Harris and Cullen, 1990). An exception to this is nigexine, a basic PLA_2 found in the venom of *Naja nigricollis*, which shows a broad cytotoxic activity (Chwetzoff *et al*., 1989*a*; Chwetzoff, 1990), in addition to its neurotoxic and direct muscle damaging effects (Rowan *et al*., 1991).

Again, receptor(s) and mechanism(s) by which these PLA_2s induce muscle cell necrosis have not yet been established. The role of enzymatic activity in myotoxicity (and other pharmacological actions, including neurotoxicity) is still not clear, despite efforts by many groups of researchers during the last 20 years (Rosenberg, 1986; Kini and Evans, 1989*b*). In many cases, chemical modification of specific amino acids have resulted in partial dissociations of the catalytic and pharmacological activities (Rosenberg, 1986, 1990). This suggests that myotoxicity, as well as other toxic actions, might be partially mediated by non-enzymatic mechanisms, contrary to the original *a priori* assumption that the toxic effects would be caused by the enzymatic activity. Based on sequence comparison data, it has been speculatively proposed that the molecular region responsible for myotoxicity would be located at amino acid residues 89-97, forming a cationic site followed by a hydrophobic stretch (Kini and Iwanaga, 1986). However, no direct evidence in support of this has yet been reported.

In addition to myotoxicity and neurotoxicity, another potent biological action of some of these PLA_2s is an anticoagulant effect (Kini and Evans, 1987). The recent achievements in the cloning and expression of some of these PLA_2s (Hodgson *et al*., 1993) should allow the construction of mutants, and clarify the understanding of their structure-function relationship.

(3b) non-neurotoxic PLA_2 myotoxins:

This group includes a growing number of enzymes found mainly in snakes of the families Crotalidae and Viperidae. The lack of neurotoxicity is reflected by their low lethal activity, which rather has been attributed to myoglobinuria and renal failure (Mebs and Ownby, 1990). Some well characterized toxins in this group are the myotoxic PLA_2s found in several *Bothrops* spp. (Gutiérrez *et al*., 1984*a*, 1986*a*, 1991*b*; Homsi-Brandeburgo *et al*., 1988; Lomonte and Gutiérrez, 1989; Heluany *et al*., 1992), *Agkistrodon* spp. (Mebs and Samejima, 1986; Johnson and Ownby, 1993), and *Trimeresurus* spp. (Mebs and Samejima, 1986; Kihara *et al*., 1992) venoms.

As mentioned above, the morphological features of muscle damage caused by these PLA_2s are similar to those observed with their neurotoxic counterparts.

Nevertheless, two notable differences with the neurotoxic PLA_2s, regarding the mode of muscle damage, open the possibility that the non-neurotoxic myotoxins may utilize a different mechanism for myotoxicity. First, some myotoxins of this group have been shown to be directly cytotoxic to immature muscle cells growing *in vitro* (Brusés *et al*., 1993; Bultrón *et al*., 1993*b*; and present work), in contrast with the neurotoxic PLA_2 myotoxins. Second, a number of natural toxin variants (or isoforms) have been found in this group, which, while clearly having PLA_2 structure, are unable to hydrolyze phospholipids due to changes in amino acid residues that are essential for the catalytic mechanism. Since these isoforms still conserve the ability to damage muscle, it is clear that the non-neurotoxic PLA_2 myotoxins can induce myonecrosis without the need of enzymatic activity. The first report of a myotoxin with PLA_2 structure, but lacking enzymatic activity, is that of *Bothrops nummifer* myotoxin (Gutiérrez *et al*., 1986*a*, 1989). Examples that followed include *B. jararacussu* bothropstoxin I (Homsi-Brandeburgo *et al*., 1988; Cintra *et al*., 1993), *B. asper* myotoxin II (Lomonte and Gutiérrez, 1989; Francis *et al*., 1991), *Trimeresurus flavoviridis* basic proteins I and II (Yoshizumi *et al*., 1990; Liu *et al*., 1990; Kihara *et al*., 1992), *Vipera ammodytes* ammodytin L (Krizaj *et al*., 1991), and *Agkistrodon contortrix laticinctus* myotoxin (Johnson and Ownby, 1993; Ownby and Li, 1993). With the exception of ammodytoxin L, in which a serine replaces the conserved aspartate residue in position 49, all other enzymatically-inactive myotoxins that have been sequenced to date possess a lysine in this position, and are therefore commonly referred to as Lys-49 PLA_2s. In addition, there are several Lys-49 PLA_2s isolated from snake venoms, on which no detailed information on their myotoxic activity is yet available, but which are probably myotoxic, on the basis of their high structural homology with known myotoxins of this group. Examples of these would be *Agkistrodon piscivorus piscivorus* K-49 -the first Lys-49 PLA_2 discovered- (Maraganore *et al*., 1984; Dhillon *et al*., 1987; Scott *et al*., 1992), and a Lys-49 PLA_2 of *Trimeresurus mucrosquamatus* (Liu *et al*., 1991). Conversely, enzymatically-inactive myotoxic PLA_2s that have not yet been sequenced, will most likely turn out to be Lys-49 variants, or variants of other critical amino acid positions. This will probably be the case of *Bothrops moojeni* myotoxins I and II (Lomonte *et al*., 1990*a*), *B. pradoi* myotoxin I (Moura-da-Silva *et al*., 1991), and *B. godmani* myotoxin II (Díaz *et al*., 1992). Indeed, work in progress on *B. godmani* myotoxin II indicates that it is a Lys-49 variant (Raghuvir Arni and José María Gutiérrez, personal communication).

Although some Lys-49 PLA_2s have been originally reported to possess a very low enzymatic activity (usually below 0.1-1.5% of that of their Asp-49 counterparts), it is now believed that Lys-49 variants are unable to perform enzymatic catalysis. Using site-directed mutagenesis of the porcine pancreatic PLA_2, it has been clearly demonstrated that the single replacement of Asp-49 by Lys-49 (or Glu-49) results in the complete abrogation of PLA_2 activity (van den Bergh *et al*., 1989). Consequently, the extremely low PLA_2 activity found in many Lys-49 preparations, and originally believed to be intrinsic (Maraganore *et al*., 1984), is most likely due to contaminating traces of enzymatically-active isoforms (i.e. Asp-49) that are difficult to remove (van den Berg *et al*., 1989; Scott *et al*., 1992).

Little progress has been achieved on the mechanism of action of this group of non-neurotoxic PLA_2 myotoxins. As in the case of their neurotoxic counterparts, the primary site of action is believed to be the plasma membrane of muscle fibers. This is suggested by the following indirect evidence: (*1*) the ultrastructural observation of early membrane ruptures or discontinuities in affected muscle fibers (Gutiérrez *et al*., 1984*b*; Johnson and Ownby, 1993); (*2*) the increased intracellular calcium concentration in affected muscle tissue (Gutiérrez *et al*., 1984*a*; 1989); (*3*) the immunodetection of myotoxins on the plasma membrane of muscle sections exposed *in vitro* (Brenes *et al*., 1987); (*4*) the ability of myotoxins to disrupt various types of liposomes (Gutiérrez *et al*., 1989; Díaz *et al*., 1991, 1992; Rufini *et al*., 1992; Bultrón *et al*., 1993*a*; Pedersen *et al*., 1993); and (*5*) the lack of inhibitory effect of endocytosis-blocking agents (ammonium chloride and chloroquine) on the cytolytic damage induced by myotoxins *in vitro* (Bultrón *et al*., 1993*b*).

Contrary to the initial working hypothesis in which muscle damage was attributed to the enzymatic hydrolysis of membrane phospholipids (Gutiérrez *et al*., 1984*a*), it has now become clear that the membrane-damaging activity of these myotoxins is exerted without the requirement of PLA_2 activity. This is demonstrated by (*1*) the ability of Lys-49 or Ser-49 variants to cause myonecrosis (Homsi-Brandeburgo *et al*., 1988; Lomonte and Gutiérrez, 1989; Krizaj *et al*., 1991; Kihara *et al*., 1992), and to disrupt liposomes (Díaz *et al*., 1991; Rufini *et al*., 1992); and in addition, indirectly supported by (*2*) antibody-neutralization studies in which myotoxins retain PLA_2 activity after inhibition of myotoxicity (Lomonte *et al*., 1992), or conversely, retain myotoxicity after inhibition of PLA_2 activity (Lomonte *et al*., 1987); and (*3*) the ability of enzymatically-active myotoxins to damage muscle *in vitro* under conditions that inhibit enzyme activity, i.e. the chelation of calcium ion by EDTA (Gutiérrez *et al*., 1986*c*; Bultrón *et al*., 1993*a*).

Although PLA_2 activity is clearly not required for membrane damage, several observations indicate that PLA_2 activity may have an enhancing role for some of these toxins. For example, *B. asper* myotoxin I (enzymatically-active) is about 2-3 times more potent in inducing myonecrosis and in disrupting liposomes, compared to the enzymatically-inactive myotoxin II (Díaz *et al*., 1991). Furthermore, when the PLA_2 activity of myotoxin I is inhibited *in vitro* by EDTA-chelation of calcium ion, the liposome-disrupting activity decreases and becomes roughly similar to that of myotoxin II, which is not affected by EDTA (Díaz *et al*., 1991). In agreement with this, myotoxin III shows a reduced muscle-damaging activity *in vitro* when its PLA_2 activity is inhibited by EDTA (Bultrón *et al*., 1993*a*). On the other hand, in the case of *Trimeresurus flavoviridis* PLA_2s, it has been recently described that Lys-49 myotoxins (basic proteins I and II) are about twice as myotoxic as the Asp-49 myotoxin (Kihara *et al*., 1992), indicating that the observations made with *B. asper* myotoxins, regarding the role of PLA_2 activity, cannot be extrapolated to all of the myotoxins in this group.

Despite these recent advances towards understanding the mode of action of this group of toxins, their membrane target(s) and the details of the membrane damage remain unknown. Especially intriguing is the molecular basis of their selectivity for skeletal muscle *in vivo*, which contrasts with their ability to affect a variety of cell types *in vitro* (Brusés *et al*., 1993; Bultrón *et al*., 1993b; and present work).

In addition to their myotoxic action, several of these PLA_2s induce edema (Gutiérrez *et al*., 1986*a*, 1986c; Selistre *et al*., 1990), even in the absence of PLA_2 activity, such as with *B. asper* myotoxin II (Lomonte and Gutiérrez, 1989; and present work). The mechanism for edema induction in the absence of PLA_2 activity is also unknown. Furthermore, when PLA_2 activity is present, these myotoxins, in similarity with various other PLA_2s (Kini and Evans, 1987) exhibit a potent anticoagulant effect *in vitro*, prolonging the recalcification time of platelet-poor plasma (Gutiérrez *et al*., 1986*c*; Díaz *et al*., 1991).

(4) Indirect myotoxic factors:

The first three groups of myotoxic factors described above, act directly upon muscle fibers to induce their degeneration and eventual necrosis. In contrast, this fourth group includes toxins that can induce local muscle damage, not via a direct action, but most likely as a secondary consequence to the damage to microvasculature, perturbations of blood flow, and development of ischemia. This category includes some hemorrhagic toxins which have been reported to induce also

muscle damage (Ownby *et al*., 1978; Fabiano and Tu, 1981; Queiroz *et al*., 1985*a*, 1985*b*; Yagihashi *et al*., 1986; Komori and Sugihara, 1988; Kamiguti *et al*., 1991). In fact, hemorrhagic toxins may be a complicating factor in the assessment of the direct muscle-damaging activity of crude venoms (Mebs and Ownby, 1990; Harris and Cullen, 1990).

The myonecrosis observed after the injection of some hemorrhagic toxins appears to develop more slowly (i.e. after about 4-6 hr) than that caused by direct myotoxins, in agreement with the concept of ischemia as a cause of muscle degeneration (Ownby, 1990). Furthermore, the morphology of muscle fibers that have been damaged by hemorrhagic toxins also appears to be different, having a more hyaline appearance (Yagihashi *et al*., 1986), also referred to as the "coagulative" type of necrosis (Homma and Tu, 1971), as opposed to the "myolytic" appearance described in the previous sections.

However, there may be exceptions to this concept of a slow development of muscle damage caused by hemorrhagic toxins. For instance, some of them have been reported to damage muscle fibres in 1 hr (Yagihashi *et al*., 1986). Another complicating observation is that some toxins can cause extensive microvessel damage, in apparent absence of skeletal muscle necrosis (Selistre *et al*., 1990). This suggests that additional factors, besides damage to microvasculature, might be necessary for the injury of muscle induced by some hemorrhagic toxins. Theoretically, among these possible concomitant factors could be (*1*) the induction of edema (with consequent tissue compression and ischemic action), (*2*) the effects on coagulation components, and (*3*) on platelets, both being critical in the development of blood flow disturbances.

Although the role of ischemia in the development of myonecrosis due to hemorrhagic toxins has been often proposed and/or assumed (Gutiérrez and Lomonte, 1989; Ownby, 1990; Ownby and Mebs, 1990; Harris and Cullen, 1990; Dos Santos *et al*,. 1992), studies providing direct evidence for such a mechanism are still lacking. These could include the evaluation of biochemical indicators of ischemia (for example lactic acid and ATP levels; Harris *et al*., 1986), and the *in vivo* analysis of vascular flow parameters (Harris and Cullen, 1990). Also, it should be possible to clearly determine (or exclude) any eventual direct action of the toxins on muscle fibers by the use of *in vitro* assays of muscle damage, such as the release of intracellular markers from gastrocnemius or other muscle preparations (Gutiérrez *et al*., 1986*b*; Melo and Suarez-Kurtz, 1987).

Several methods are available for the evaluation of myotoxic activity of venoms and toxins, both *in vivo* and *in vitro*. A simple technique has been proposed for the

quantification of the "necrotizing" activity of venoms (Theakston and Reid, 1983) based on an intradermal injection and measurement of skin lesion after 3 days, but this assay does not evaluate myonecrosis, and it is not clear what type of venom components it is able to detect. Histological analysis of injected muscle tissue is undoubtedly the reference method, and can be made quantitative (Ownby *et al*., 1982; Kouyoumdjian *et al*., 1986; Ownby and Colberg, 1988; Preston *et al*., 1990; McLoon *et al*., 1991). Nevertheless, quantitative histology requires considerable work and equipment, and for this reason, many studies have utilized histological evaluation only in a qualitative manner. As an alternative, methods based on the measurement of biochemical markers of skeletal muscle damage have been utilized. These include the quantification of specific intracellular enzymes released after cell injury, either by measuring their increased activity in plasma (or serum), or their decreased intracellular content in the affected muscle tissue. Among these enzymes, creatine kinase (CK; EC 2.7.3.2) has been frequently utilized. This enzyme occurs in three electrophoretically distinct isoforms, MM, MB, and BB, which are preferentially expressed in skeletal muscle, cardiac muscle, and central nervous system, respectively (Raphael, 1983). When total CK quantification is utilized in the study of skeletal muscle damage induced by a given venom or toxin, it is important to verify that its increase is due to the CK-MM isoenzyme (Nakada *et al*., 1980, 1984; Gutiérrez *et al*., 1986*a*, 1986*c*; Chaves *et al*., 1989). Moreover, pharmacokinetic parameters such as the distribution and plasma half-life of the enzyme may significantly influence the results. Therefore, it is also important to select an optimal sampling time in these determinations (Gutiérrez *et al*., 1980; Ownby *et al*., 1982). Time-course experiments of the CK release after myonecrosis induced by non-neurotoxic PLA_2 myotoxins (Gutiérrez *et al*., 1984*a*, 1991*b*; Lomonte and Gutiérrez, 1989; Díaz *et al*., 1992), or their corresponding crude venoms (Gutiérrez *et al*., 1980, 1984*a*; Melo and Suarez-Kurtz, 1988; Chaves *et al*., 1989), have shown that, in general, the plasma peak occurs between 1 and 3 hr after injection, rapidly declining thereafter.

The use of CK, or other enzyme markers, as estimators of muscle necrosis may not necessarily be appropriate for all types of muscle-damaging toxins, since different cytopathogenic mechanisms may participate in each case, as discussed above. It is possible that CK, despite being an enzyme of considerable size (81 Kd), may be released in reversible stages of cell injury, for example, as reported in the case of myotoxin *a*, of the group of small myotoxins (Ownby *et al*., 1982). Suarez-Kurtz (1982) has shown that CK can be released from frog skeletal muscle by osmotic changes, in apparent absence of irreversible cell damage, as judged by

electrophysiological criteria (quantification of muscle twitch amplitude). In the case of *Notechis scutatus* venom, containing the presynaptically neurotoxic PLA_2 notexin, the correlation of muscle necrosis to increased plasma CK levels was not satisfactory (Preston *et al*., 1990), although in this study the correlation was analyzed at 24 hr, a time point in which the plasmatic CK levels were declining. Nevertheless, at least with the group of non-neurotoxic PLA_2 myotoxins, or venoms containing these toxins, a fair correlation appears to exist between the amount of venom injected and the plasma CK increase (Nakada *et al*., 1980; 1984; Gutiérrez *et al*., 1980; Mebs *et al*., 1983). This might be a reflection of the type of mechanism utilized by such myotoxins, which, as discussed above, presents some subtle differences with that of neurotoxic PLA_2s, and more obvious differences with that of small myotoxins. More quantitative studies, using different venoms and myotoxin types, are needed to analyze the validity of CK measurements in the assessment of myonecrosis. In addition, other methods, ideally combining simplicity and reliability, should be developed and evaluated.

Ex vivo, muscle preparations such as the mouse gastrocnemius or the rat extensor digitorum longus muscles, have been utilized in the determination of myotoxic activity, using the release of CK into the bathing solution as a marker of cell damage (Gutiérrez *et al*., 1986*c*; Melo and Suarez-Kurtz, 1987, 1988). Some advantages of these *ex vivo* systems over *in vivo* tests are the lack of influence of enzyme distribution and half-life, the possibility to control variables in the bathing solution, and the reduction of experimental animals by half, if muscles from both extremities of a single individual are utilized.

In vitro, cell cultures of skeletal muscle myoblasts, myotubes, or even other cell types, might have an interesting potential as models for the assessment of myotoxic activity, at least for some myotoxin types. This area of research is still relatively new and not fully exploited. If these cell culture systems prove to correlate well with *in vivo* results, they could become invaluable simple substitutes of live animals for the study of some myotoxins and their neutralization by antibodies or other types of blocking molecules.

c. Edema-inducing toxins

Many types of snake venoms contain potent inflammatogenic factors that induce a rapid and drastic edema response (Bonta *et al*., 1979; Tan and Saiffudin, 1990). One important consequence of edema is tissue compression (Garfin *et al*., 1985) and the resultant enhancing effect on the ischemic conditions that may arise at the site of the

bite, contributing in this way to the development of local tissue damage. In addition to the local consequences, an edema of considerable magnitude will contribute to the hypovolemic and hypotensive effects of venom that lead to cardiovascular shock.

Edema-inducing components in snake venoms are biochemically heterogeneous, and may range from preformed biogenic amines of the histamine type (Tilmisany *et al*., 1986) to small peptides or proteins such as phospholipases A_2 (Vishwanath *et al*., 1985, 1987, 1988; Marshall *et al*., 1989; Teng *et al*., 1989; Lomonte and Gutiérrez, 1989; Wang and Teng, 1990*a*, 1990*b*, 1991, 1992; Liu *et al*., 1991; Tan *et al*., 1991; Ferrer and Moreno, 1992; Nair *et al*., 1993), esterases (Ohtani and Takahashi, 1983; Oguchi *et al*., 1986; Teng *et al*., 1989; Wang *et al*., 1991), proteases (Teng *et al*., 1989), kinin-releasing enzymes (kallikreins, kininogenases; Mebs, 1970; Vargaftig *et al*., 1974; Bjarnason *et al*., 1983; Samel *et al*., 1987; Bailey *et al*., 1991), and lectins (Lomonte *et al*., 1990*b*).

The edema induced by venom PLA_2s has been intensively studied from the pharmacological point of view, perhaps because considerable evidence has accumulated in recent years to support the inflammatogenic role of human non-pancreatic (extracellular) PLA_2 in a variety of pathological conditions (Pruzanski and Vadas, 1991), despite some still controversial observations (Morgan *et al*., 1993). As this human enzyme shows close structural homology with those obtained from venom secretions, especially group II PLA_2s (Davidson and Dennis, 1990), the latter enzymes, which are more readily obtainable, are being used as models for the assay of potential inhibitors of inflammation (Marshall *et al*., 1989).

The majority of edema-inducing venom components probably promote inflammation indirectly, by releasing or inducing potent autacoids. As an example, many venoms, in all families of snakes, have been shown of being capable of releasing histamine from animal tissues (Rothschild and Rothschild, 1979), which would be responsible for the immediate phase of increased vascular permeability via H_1 receptors on endothelial cells (Trowbridge and Emling, 1989; Atkinson *et al*., 1992), among other disturbances.

Later stages of edema are believed to be induced by several types of mediators, including bradykinin, prostaglandins, and leukotrienes, acting in a complex interrelated manner (Trowbridge and Emling, 1989). Bradykinin, a potent hypotensive, pain-inducing, and microvascular permeability-increasing mediator (Kozin and Cochrane, 1992), can be generated in response to venoms (Mebs, 1970, 1990; Rothschild and Rothschild, 1979; Bonta *et al*., 1979). Prostaglandins and leukotrienes, which probably mediate the delayed, prolonged phase of vascular

permeability increase (Trowbridge and Emling, 1989), have also been shown to be induced by some venoms (Rothschild and Rothschild, 1979). Anaphylotoxins C3a and C5a of the complement system, which have a potent action on vascular permeability, are known to be released by the "cobra venom factor" of the *Naja* spp. snakes (Vogt, 1990).

All these findings suggest that the edema induced by snake venoms is multifactorial in origin, and therefore, that no single inhibitor or drug would be expected to counteract it (Bonta *et al*., 1979; Detrait and Jacob, 1988; Trebien and Calixto, 1989). The diversity of mechanisms participating in the induction and maintenance of edema is observed even at the level of single toxin components (Calhoun *et al*., 1989; Marshall *et al*., 1989; Chen *et al*., 1990; Wang *et al*., 1991). As an example, "TMV F-IV", an edema-inducing toxin purified from *Trimeresurus mucrosquamatus* venom, has been shown to act by the release of histamine, serotonin, arachidonate metabolites PGE_2 and LTB_4, platelet-activating factor (PAF), involving the participation of both mast cells and neutrophils (Wang *et al*., 1991). Even in studies with purified toxins, their relative contribution to the overall edema-inducing activity of the crude venom has generally not been determined, and the purified components may show lower activity than the crude venom.

Among the various mechanisms described in the induction of edema by purified venom components are: mast cell degranulation with release of histamine/serotonin (Chiu *et al*., 1989; Calhoun *et al*., 1989; Cirino *et al*., 1989; Wang and Teng, 1990*a*; Moreno *et al*., 1992; Lloret and Moreno, 1993), attraction of polymorphonuclear neutrophils (Wang *et al*., 1991) with superoxide radical formation (Wang and Teng, 1991, 1992), induction of prostaglandins and leukotrienes (LTB_4) in mast cells (Wang *et al*., 1991), release of bradykinin (Cohen *et al*., 1970; Vargaftig *et al*., 1974; Teng *et al*., 1992), release of a vasoactive peptide different from bradykinin (Oguchi *et al*., 1986), induction of endothelium-derived relaxing factor (now identified as nitric oxide; Kolb and Kolb-Bachofen, 1992) via bradykinin stimulation of endothelium (Cirino *et al*., 1991), potentiation of bradykinin activity by peptide inhibitors of angiotensin-converting enzyme (Ferreira *et al*., 1992), and release of slow-reacting substance (now identified as LTC_4 and its metabolites LTD_4 and LTE_4; Lam and Austen, 1992) (Huang, 1984).

The observation that antivenoms commonly utilized for the serotherapy of snakebite show a very limited efficacy in the neutralization of the edema-inducing activity of venoms (Gutiérrez *et al*., 1981, 1986*e*; Lomonte, 1985; Rojas *et al*., 1987),

suggests that more efforts should be directed towards its inhibition by combinations of drugs.

Experimental quantification of edema is usually performed in the paws of mice or rats, by measurement of simple parameters such as footpad weight, volume, thickness, or tissue dye accumulation (Yamakawa *et al*., 1976; Ponton *et al*., 1983; Van Loveren *et al*., 1984; Neves *et al*., 1993). The biological potency is expressed in arbitrary units such as the "Minimal Edema-forming Dose" (MED), defined as the amount of substance that induces 30% edema at a given time. In a comparison of the potencies of 24 venoms from different families of snakes, MEDs determined at 1 hr ranged between 0.16 and 3.41 µg/mouse paw (Tan and Saifuddin, 1990).

Snakebite serotheraphy and neutralization of tissue-damaging toxins

Heterologous immunoglobulin preparations with antivenom activity have been used for the treatment of snakebites since the pioneer work of Calmette, and Phisalix and Bertrand, in 1894 (reviewed by Grabar, 1986; Ménez, 1991). Industrial production involves the active immunization of animals, mainly equines, with sublethal increasing doses of crude venom(s), usually administered with adjuvants. When antibody levels have reached a predetermined neutralizing titer (against the lethal effect of the venom), plasma is obtained and processed for the purification of immunoglobulins. The degree of purification may vary widely among different producers, ranging from almost no purification (i.e. the use of total plasma as antivenom), to intermediate (i.e., ammonium sulfate fractionation of globulins, caprylic acid purification of total IgG), or high (i.e., affinity-purified antibodies) (Carroll *et al*., 1992). Some antivenoms are based on undigested antibody molecules, while others consist of $F(ab')_2$ or Fab fragments (Smith *et al*., 1992). Antivenoms can be monovalent, that is, prepared with the venom of a single species, or polyvalent, prepared with a mixture of venoms from more than one species. In the latter case, venom mixtures used as immunogens are grouped on the basis of their antigenic relationships (cross-neutralization characteristics), and geographical distributions of the relevant snake species in a given country or region of the world (Theakston and Warrell, 1991).

Not all snakebites require the indiscriminate use of an antivenom, depending on factors such as species of snake involved and the amount of venom injected. However, in severe envenomations, especially by some highly poisonous species, the use of a good quality antivenom is crucial for the clinical outcome (Reid *et al*., 1963;

Russell *et al*., 1975; Warrell *et al*., 1980; Reid and Theakston, 1983; Ho *et al*., 1986*b*; Benbassat and Shalev, 1993).

Antivenoms are carefully controlled for their neutralizing potency against the lethal action of venoms in mice. However, less information is available on their ability to neutralize other venom actions, among them, the tissue-damaging activities. A World Health Organization committee stressed the relevance of this point, in discussing the standardization of antivenoms (W.H.O., 1981). Given the complexity of venom composition, added to the variability of the immune response in genetically distinct animals, antivenoms are not expected to contain equal amounts of antibodies to all venom components, or equal neutralizing potencies against all of them. Furthermore, with the possible exception of some highly neurotoxic venoms, in which the main lethal toxins have been identified, the lethal action of the majority of snake venoms is poorly characterized, as many factors are probably contributing to it in a synergistic way.

Several studies have been focused on the neutralizing capacity of antivenoms towards the local tissue-damaging activities of venoms (Homma and Tu, 1970; Russell *et al*., 1973; Harris and Johnson, 1978; Gutiérrez *et al*., 1981, 1985, 1986*e*, 1987, 1991*a*; Ownby *et al*., 1983*a*, 1984*a*, 1984*b*, 1985, 1986; Lomonte, 1985; Rojas *et al*., 1987; Ownby and Colberg, 1988; Chaves *et al*., 1989; Lomonte *et al*., 1987, 1990*d*; Mebs *et al*., 1988; Antunes *et al*., 1989; Kornalík and Taborska, 1989; Laing *et al*., 1992; Dos Santos *et al*., 1992). In general, experiments have been performed using two basic modalities, which provide different, but complementary, information (Gutiérrez *et al*., 1990*a*). The first is the preincubation modality, in which an appropriate amount of the venom (selected on the basis of dose-response analysis) and varying amounts of the neutralizing agent (antivenom in this case) are mixed, incubated, and then assayed *in vivo* for the specific toxic activity. The second modality consists of the independent administration of venom and antivenom *in vivo*, mimicking the actual situation of serotherapy. The first approach may seem irrelevant from a practical point of view, nevertheless, it provides clear information about the presence and potency of neutralizing antibodies in the antivenom. The second approach evaluates the true neutralizing effectiveness of these antibodies with respect to a specific venom action during *in vivo* serotherapy, and is influenced by the pharmacokinetics of both antibodies and venom components, and the speed of toxic effects. Therefore, a negative result (lack of neutralization) in the independent administration assay, has two possible explanations: (*1*) the lack of neutralizing antibodies in the antivenom; or (*2*) the ineffectiveness of neutralizing antibodies,

despite their presence, due to pharmacokinetic considerations. Clearly, the results provided by the preincubation modality provide the basis to distinguish between these two alternatives.

In general, experimental evaluations of the neutralizing ability of antivenoms towards the local tissue-damaging actions of venoms (hemorrhage, myonecrosis, and edema), have shown only partial inhibitions, even if antivenoms were administered within the first minutes after the venom. This is in agreement with clinical observations in which administration of antivenoms seems to have little influence on the development of local effects (Reid *et al*., 1963; Warrell *et al*., 1976*b*), probably due to the delay in injection, as time is the most critical factor. One example of this problem is the hemorrhage induced by the venom of *Bothrops asper*: the corresponding antivenom is extremely efficient in neutralizing this effect by preincubation tests, but hemorrhage is only partially prevented by (early) independent administration of antivenom in mice (Gutiérrez *et al*., 1981, 1985). In other cases, however, it has been clearly shown that antivenoms may lack, or have extremely low amounts of, neutralizing antibodies against the relevant toxins for a given effect. This would be the case of a polyvalent antivenom utilized in the United States of America, which was shown to lack significant amounts of antibodies to myotoxin *a* and crotamine (Ownby *et al*., 1979, 1983*b*) despite that their immunogenicity was demonstrated by immunization of rabbits. Another example of a commercially available antivenom utilized in Latin America, lacking antibodies to the PLA_2 myotoxins found in many *Bothrops* spp. venoms, has been reported (Lomonte *et al*., 1991). The reason for this is unclear, since three other antivenoms from different producers had high antibody titers to these toxins.

Even if the rapid development of tissue damage by venoms is a major obstacle to the neutralizing efficiency of antivenoms, the aim of obtaining high levels of neutralizing antibodies against locally-acting toxins should not be neglected. The partial neutralizations observed at the experimental level obviously imply significant benefit to the patients. Moreover, the presence of appropriate amounts and specificities of antibodies in antivenoms should reduce the spreading of toxins (and damage) to distant sites, as well as their half-lives in the body.

In addition to the conventional neutralization of toxins with antibodies, potential alternative inhibitors of venom actions, natural or synthetic (Nakagawa et al., 1982; Mors *et al*., 1989; Crosland, 1989, 1991; Robeva *et al*., 1991; Martz, 1992), should be investigated. A precise understanding of the molecular sites and mechanisms of toxin

neutralization should help in the design of improved blocking agents for therapeutic use in the future (Ménez, 1991).

Cytokines in inflammation

When an injurious agent -chemical, physical, or infectious- affects the organism, a complex response is triggered, involving the participation of many cell types and their products. Essential to this inflammatory response is the participation of soluble messengers that allow the proper communication and regulation of the activities of the different cell types involved. It is now well established that one important group of mediators produced in response to injurious agents is constituted by the family of cytokines (Cerami, 1992). Although these molecules are highly pleotropic, participating in numerous diverse physiological processes, cytokines more typically involved in inflammatory reactions include IL-1, IL-6, IL-8, TNF-α and IFN-γ (Abbas *et al*., 1991). The biological activities of these cytokines in relation to inflammation are briefly summarized below.

Interleukin 1 (IL-1) is produced mainly by mononuclear phagocytes, and occurs in two forms (α and β), which show low homology, but bind to the same receptor. IL-1α is mainly found associated to membranes and IL-1β in circulation. In relation to inflammation, this cytokine can induce the synthesis of enzymes for prostaglandin production (in turn mediating fever), synthesis of IL-6 and IL-8 by endothelial cells, and their activation to promote coagulation, expression of adhesion molecules and leukocyte adhesion (Abbas *et al*., 1991; Dinarello, 1992). A natural inhibitor for this cytokine has been described, the IL-1 receptor antagonist (Seckinger *et al*., 1987; Tilg *et al*., 1993).

IL-6 is produced by many cell types, among them macrophages, endothelial cells, fibroblasts, and T lymphocytes. A known stimulus for its synthesis is IL-1, and to a lesser extent tumor necrosis factor (TNF-α). Bradykinin has been reported to induce IL-6 production in fibroblasts (Vandekerckhove *et al*., 1991). IL-6 does not cause vascular thrombosis or the tissue injury seen in response to TNF-α. A main action of IL-6 is the induction of the acute-phase response, together with TNF-α and IL-1, by affecting the synthesis of several specific proteins in hepatocytes (Mackiewicz *et al*., 1988; Castell *et al*., 1989; Lewis *et al*., 1992). Histamine can upregulate IL-6 production and enhances the production of fibrinogen and C3 induced by IL-6 in the liver (Falus and Merétey, 1992). IL-6 also induces the maturation of megakaryocytes to increase platelet numbers (Hirano *et al*., 1990) and stimulates the division of myoblasts (Austin and Burgess, 1991), among several other actions.

IL-8 can be produced by all leukocytes, platelets, and endothelial cells, in response to external stimuli such as lipopolysaccharide, or to other cytokines (IL-1 and TNF-α). It is a chemotactic and activating factor for neutrophils and, to a lesser extent, for eosinophils, basophils, and lymphocytes (Rot, 1992). A related molecule in this IL-8 family of peptides, monocyte chemotactic protein-1 (MCP-1) seems specific for mononuclear phagocytes (Abbas *et al*., 1991).

TNF-α is mainly produced by macrophages. It stimulates the production of IL-1 and IL-6, and participates in the induction of the acute-phase response, as well as being responsible for cachexia and shock when high amounts are produced (Sáez-Llorens and Lagrutta, 1993). An immediate fall in circulating neutrophils, followed by leukocytosis, lymphopenia, and IL-6 increase, is observed in healthy humans after TNF-α injection (Dinarello, 1992). TNF-α also induces the expression of adhesion molecules on endothelial cells (Cramer, 1992), and the synthesis of platelet-activating factor (PAF), and prostaglandins PGI_2 and PGE_2 (Dinarello, 1992).

Interferon gamma (IFN-γ) is produced mainly by T lymphocytes. Among its many immunomodulating activities, it is a potent activator of mononuclear phagocytes, and, to some extent, of neutrophils. It promotes lymphocyte adhesion to endothelial cells, and potentiates the effects of TNF-α on this cell type (Abbas *et al*., 1991).

Several of the cytokines involved in the process of leukocyte migration into injured tissues are believed to be immobilized and maintained at high concentrations at or near the vessel wall, probably on endothelial cell surfaces, via the interaction with their different proteoglycans (Tanaka *et al*., 1993). Cytokines such as TNF-α, IL-1, and transforming growth factor beta (TGF-β), increase the synthesis of heparan sulfate proteoglycan in endothelial cells. This glycosaminoglycan plays a role in the binding of growth factors for endothelial cells, as well as in maintaining their permeability barrier function and anticoagulant properties, and is rapidly released from the endothelial lining of blood vessels during inflammation (Ihrcke *et al*., 1993).

Despite the known importance of cytokines in the development and regulation of inflammatory reactions, both acute and chronic, there are no studies available on their possible involvement in snake venom poisoning.

Heparin and snake venoms

The idea of using heparin in the management of snakebites was proposed almost 50 years ago (Ahuja *et al*., 1946; Ahuja and Singh, 1954). Since then, a number of clinical and experimental studies evaluating its possible beneficial effects on the

severe coagulation disturbances, such as the intravascular coagulation syndrome, caused by some snake venoms have been reported, with controversial conclusions (De Vries *et al*., 1963; Tinoco, 1972; Weiss *et al*., 1972; Warrell *et al*., 1976*a*; Schaeffer *et al*., 1986; Shah *et al*., 1986; Lwin *et al*., 1989; Than *et al*., 1989; Estrade *et al*., 1989; Dempfle *et al*., 1990; Tibballs and Sutherland, 1992; Swe *et al*., 1992).

Heparin, due to its strongly polyanionic nature, can interact with many molecules possessing cationic sites (Zhou *et al*., 1992), and has been used as a tool in the study of the activities of different kinds of molecules, including snake toxins (Vaccari and Fregnan, 1955; Condrea and De Vries, 1964; Higginbotham, 1965; Lee *et al*., 1970; Ho *et al*., 1986*b*; Melo *et al*., 1993). Heparin-binding proteins include a variety of growth factors, proteins of the extracellular matrix, proteins involved in lipid metabolism, serine protease inhibitors, viral proteins, and enzymes (Zhou *et al*., 1992). Among these, the interaction with PLA_2s, either affecting their enzymatic activity (Franson *et al*., 1974; Diccianni *et al*., 1990, 1991) or not (Condrea and De Vries, 1964; Avila and Convit, 1976; Ho *et al*., 1986*a*; Horigome *et al*., 1987), is of interest in snake venom research, as these enzymes possess several pharmacological/toxic actions, as discussed above (Rosenberg, 1990).

It has been reported that heparin can neutralize the myotoxic activity of the venom of *Bothrops jararacussu* (Melo and Suarez-Kurtz, 1988) or, very recently, one of its myotoxic components, bothropstoxin, of the group of non-neurotoxic PLA_2s (Melo *et al*., 1993). These findings suggest that, apart from its still debated effects on the coagulation disturbances caused by some venoms, heparin might have an additional usefulness in the treatment of snakebites, as a direct neutralizing molecule for particular venom components.

Heparin is a glycosaminoglycan mainly composed of repeating units of hexuronic acid (either iduronic acid or glucuronic acid) and glucosamine, carrying a high number of sulfate substitutions, which are responsible for its strong negative charge. Sulfate groups may be found in five different locations: linked to the amino groups of the glucosamine moiety (N-sulfate), and as ester sulfate (O-sulfate) on C-6 and C-3 of glucosamine, and on C-2 of iduronic and glucuronic acid (Rodén *et al*., 1992). Owing to the variability in sulfate substitution, heparin, like other glycosaminoglycans, displays considerable sequence heterogeneity both within and between different chains (Kjellén and Lindahl, 1991). Heparin is polydisperse, with a molecular weight ranging from 5 to 40 kD, and an average of 13-15 kD (Linhardt *et al*., 1992). Only about one third of the molecules of standard heparin preparations contain the specific pentasaccharide sequence required to interact with antithrombin

and exert anticoagulant activity (Rodén *et al*., 1992). Thus, it is possible to fractionate heparin by affinity chromatography on immobilized antithrombin, in order to obtain preparations with very low or no anticoagulant action ("low affinity" heparin), and full anticoagulant action, respectively.

Heparin is produced only by mast cells, being found in their secretory granules. Virtually all other cell types synthesize heparan sulfates, which show similar fundamental compositions and structures to heparins, but also substantial quantitative differences. There is more glucuronic acid and, consequently, less iduronic acid in heparan sulfate than in heparin, the N-acetyl content is higher and the N-sulfate content lower, and the degree of O-sulfation is lower (Rodén *et al*., 1992). These characteristics give heparan sulfate a lower overall charge density in comparison to heparin, although clusters of N-sulfated, more extensively modified, sequences, are still present in the former (Kjellén and Lindahl, 1991).

AIMS OF THE STUDY

1.To characterize the local tissue damage and inflammatory reactions that develop after experimental injection of snake venoms and isolated toxins, in a mouse model.

2.To develop and evaluate experimental *in vivo* and *in vitro* methods to quantify cell and tissue damage caused by venoms and toxins, as well as their neutralization by antibodies or drugs.

3.To characterize the ability of heparins to interact with, and neutralize, myotoxins from snake venoms.

MATERIALS AND METHODS

Snake venoms

The venoms of *Bothrops asper* (Pacific variety) and *Micrurus nigrocinctus nigrocinctus* were pools obtained from at least 30 specimens of each species, collected in Costa Rica, and kept at the serpentarium of Instituto Clodomiro Picado (University of Costa Rica). Immediately after collection, venoms were centrifuged to remove debris, lyophilized, and stored at -20°C. The venoms of *Vipera berus* (Russian origin), *Agkistrodon piscivorus piscivorus*, *Bothrops jararacussu*, *Crotalus durissus terrificus*, *Crotalus viridis viridis*, *Naja naja atra*, and *Trimeresurus flavoviridis* (unspecified geographical origins) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A).

Purified toxins and synthetic peptide

Myotoxin II was purified from the venom of *B. asper* by two cycles of ion-exchange chromatography on CM-Sephadex C-25 (Pharmacia, Sweden) as described by Lomonte and Gutiérrez (1989). Myotoxin III (Kaiser *et al*., 1990) and hemorrhagic metalloproteinase BaH-1 (Borkow *et al*., 1993) purified from the same venom, were kindly provided by Dr. J.M. Gutiérrez (University of Costa Rica).

A 26-mer peptide corresponding to the amino-terminal sequence of myotoxin II (Ser-Leu-Phe-Glu-Leu-Gly-Lys-Met-Ile-Leu-Gln-Glu-Thr-Gly-Lys-Asn-Pro-Ala-Lys-Ser-Tyr-Gly-Ala-Tyr-Gly-Cys) with native endings was synthesized by Chiron Mimotopes Ltd. (Victoria, Australia) using Fmoc strategy (Valerio *et al*., 1993) and had an estimated purity of 96% by HPLC on a LiCrosphere 100RP-18 column. The amino acid sequence was based on data from Francis *et al*. (1991).

Antivenoms and other antibodies

Equine polyvalent crotalid antivenom (batches P-183 and 203LQ) and anti-*Micrurus nigrocinctus* antivenom (batch 207A89LF) for human use were prepared at the Instituto Clodomiro Picado (University of Costa Rica), as described by Bolaños and Cerdas (1980).

A rabbit antiserum against *Vipera berus* venom was raised by injecting 1 mg of venom, emulsified with Freund's complete adjuvant, by the i.m. route. On days 21, 36, and 51, respectively, additional 1 mg doses were given using sodium alginate as a vehicle. Serum was obtained 14 days after the last booster

immunization. The antibody titer and reactivities were determined by enzyme-immunoassay and immunoblotting, respectively, as described below.

MAb-3, a mouse IgG_1 neutralizing monoclonal antibody against *B. asper* myotoxins (Lomonte and Kahan, 1988) was prepared as ascitic fluid in pristane-primed BALB/c mice, and partially purified by ammonium sulfate precipitation. Its concentration was determined by radial immunodiffusion (Mancini *et al*., 1965) using a mouse IgG_1 standard (Sigma).

Heparins and other glycosaminoglycans

Standard heparin (5000 IE/ml), heparin with low affinity for antithrombin (LA-heparin; 7 IU anti-factor Xa/mg, M_r 15 kD), and low molecular weight heparin (Fragmin®, 25000 IE/ml, M_r 5 kD) were kindly provided by Kabi Pharmacia (Sweden). Heparan sulfate (from bovine intestinal mucosa, H-7641) and a series of heparin-derived disaccharides were obtained from Sigma. A low-sulfated heparan sulfate preparation (~0.6 sulfate group/disaccharide unit) from human aorta (Iverius, 1971) was kindly provided by Dr. W. Murphy (University of Monash, Australia). Chondroitin sulfate (from bovine cartilage) and dermatan sulfate (from pig intestinal mucosa) were prepared as described by Lycke *et al*. (1991). Heparin from pig intestinal mucosa (Lindahl *et al*., 1965) was radiolabeled by [^{3}H]acetylating free amino groups with labeled acetic anhydride (Höök *et al.*, 1982). Even-numbered heparin oligosaccharides were generated by partial deaminative cleavage with nitrous acid (pH 1.5; Shively and Conrad, 1976; Pejler *et al*., 1988), followed by reduction with NaB^3H_4 (Amersham, U.K.). Labeled oligosaccharides were separated by repeated gel filtration on Sephadex G-50 into even-numbered species, from 4- to 14-saccharides (Lane *et al*., 1984). Selectively O-desulfated heparins (2-O-desulfated and 6-O-desulfated) were kindly given by Drs. A. Naggi and G. Grazioli (Istituto G. Ronzoni, Milano, Italy). N, O-desulfated heparin was prepared as described by Nagasawa *et al*. (1977). This preparation was subjected to either complete re-N-sulfation (Lloyd *et al*., 1971) or complete re-N-acetylation (Danishefsky *et al*., 1965).

Venom lethal activity

Lethal potency was determined in Swiss mice (16-18 g) using groups of five animals per dose (i.p., in 500 μl of phosphate-buffered saline; PBS). Deaths were scored after 48 hr, and the lethal dose 50% (LD_{50}) was calculated by probit

analysis using the computer program described by Trevors (1986). Animal experiments were performed in accordance with ethics permit #53-92.

Hemorrhagic activity

Hemorrhage induced by venoms or toxins was quantified in mice (18-22 g), 2 hr after i.d. injection (in 100 µl of PBS), by measuring the diameter of the hemorrhagic spot formed in the internal side of the skin (Kondo *et al*., 1960).

Edema-forming activity

Edema was quantified in the footpad of mice (22-28 g) by measuring the thickness increase with a low-pressure spring caliper (Oditest, H.C. Kröplin, Germany) (Van Loveren *et al*., 1984). Venoms or toxins were injected s.c. in a total volume of 50 µl of PBS.

Myotoxic activity: plasma creatine kinase method

Venoms or toxins were injected i.m. into mice (gastrocnemius muscle) in a volume of 50-100 µl of PBS. Control animals received PBS alone. After 3 hr (Gutiérrez *et al*., 1984*a*, 1991*a*), serum levels of creatine kinase (CK; EC 2.7.3.2) were determined by a colorimetric assay (Sigma No.520). Activity was expressed as units/ml, one unit being defined as the phosphorylation of one nanomole of creatine per min at 25°C.

Myotoxic activity: muscle residual creatine kinase method

Mice injected i.m. (as above) were sacrificed after 24 hr, and both gastrocnemius muscles (injected and contralateral control) were removed, weighed and homogenized in 4 ml of PBS containing 1% Triton X-100, using a Brinkmann PT10/35 homogenizer (Polytron, Switzerland). After centrifugation at 1000 *g* for 10 min, each supernatant was diluted 1:40 with distilled water and 7 µl of this dilution were assayed for CK activity (as above). The activity of each supernatant was divided by its corresponding muscle weight. The muscle residual CK activity was expressed as a percentage of the corresponding control (contralateral) muscle value.

Myotoxic activity: muscle residual MTT-reduction method

Muscle homogenates were prepared exactly as above, from mice injected i.m. in the gastrocnemius. Then, 1 ml of each supernatant (undiluted) was mixed with 0.2 ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)

solution (2.5 mg/ml PBS) in a 1.5 ml microfuge vial and incubated in the dark at 37° C for 90 min. Final absorbances were determined at 570 nm against a 690 nm reference (Denizot and Lang, 1986) on a Shimadzu UV-160 spectrophotometer. Each absorbance value was divided by its corresponding muscle weight. The muscle residual MTT-reducing activity was expressed as a percentage of the control (contralateral) muscle value.

Myotoxic activity: quantitative histological method

Venom was injected by i.m. route as above. After 24 hr, the injected gastrocnemius muscle was removed, cut transversally in the middle portion, and immediately immersed in Duboscq-Brasil fixative (10% formalin, 50% ethanol, 6.5% acetic acid, 0.45% picric acid), then dehydrated in ethanol and embedded in paraffin. The orientation of the tissue was carefully controlled during embedding in order to obtain cross sections. Transverse sections (5-8 μm) were stained with a modified Masson trichrome stain (Arce, 1986). Video recordings encompassing the whole area of each section were made, and the total number of necrotic and normal muscle cells were counted for each sample. A "Necrosis Index" was calculated as number of necrotic cells/total number of cells.

For qualitative histological evaluations, tissue samples were fixed in 4% paraformaldehyde, 0.1 M cacodylate buffer (pH 7.4), decalcified (if pertinent), embedded in parafin, and stained with hematoxylin-eosin.

Intravital microscopy

Local tissue damage induced by *B. asper* venom or myotoxin II was analyzed by intravital microscopy. Male C57BL mice (ALAB, Sweden) were anaesthetized and placed in supine position on a water-heated bed. The cremaster muscle was surgically exposed, pinned onto a special heated observation platform, and covered with a thin Mylar® sheet (6 μm) to prevent evaporation. Tissue reactions were observed in a Leitz vital microscope, equipped with a color TV-camera (WV CD130; Panasonic, Japan), a black and white low light-level TV-camera for fluorescence microscopy (WV-1900/G; Panasonic), a VCR (NV-FS88 HQ; Panasonic), a video timer (VTG-33; FOR-A Co. Ltd, Japan), a 19" video color monitor (Panasonic), a VCR image enhancer (Detailer II; Vidicraft, Japan), and a video copy processor (P66E, Mitsubishi, Japan). Dry objectives x2.5;0.08, x6;0.18, x20;0.32 and water immersion objectives x55;084 and x75;1.0 were used in combination with x12.5 eye-pieces. A Leitz Ploemopak illuminator and a Xenon

burner (150 W) were used for incident light fluorescence microscopy to study microvascular plasma leakage. This was visualized by i.v. injection of 0.10-0.15 ml of FITC-dextran (25 mg/ml PBS; mol wt 147,800; Sigma) given 5 min before the local application of venom or myotoxin, through the cannulated left femoral vein. For studies on plasma leakage, venom or myotoxin were mixed with 1% agarose-saline at 50°C, and immediately 20 µl (of 2.5 mg/ml final concentration) were spread over a small piece of plastic film (Gelbond®, Pharmacia). The gel was gently placed on the tissue after moving the Mylar® to the side so that it covered about 2/3 of the muscle, serving as a control area. After 5 min exposure to the gel, the muscle was flushed with warm saline and covered again. The transparency of the gel allowed continuous observation of the microcirculation during the period that it was in contact with the muscle. For the rest of the experiments, the whole of the muscle was exposed to venom or myotoxin by lifting off the Mylar® and dripping 20 µl of a solution of either substance (2 mg/ml) on the tissue. The exposure time varied from 5-30 min, during which the muscle was covered, before being flushed with warm saline and once again covered for the following observation period (30 min).

Electron microscopy

At the end of some of the intravital microscopy experiments, the cremaster muscle was fixed for 5 min with a solution consisting of 2.5% glutaraldehyde, 1% paraformaldehyde and 0.01% sodium azide in 0.05 M sodium cacodylate, pH 7.2, and then removed. The specimens were further fixed in the same solution overnight, followed by post-fixation in 1% OsO_4 in 0.1 M sodium cacodylate, dehydration and infiltration with epoxy resin (Agar 100) according to routine procedures. Ultrathin sections were cut with diamond knives in a Reichert Ultracut E microtome and contrasted with lead citrate and uranyl acetate before examination in a Zeiss CEM 902 electron microscope.

Quantification of cytokines

The release of proinflammatory cytokines, after the injection of venoms or toxins into mice, or after the *in vitro* exposure of their splenic cells to venoms, was evaluated by either immunoassay or bioassay.

TNF-α was quantified by cytotoxicity on the WEHI 164-clone 13 murine fibrosarcoma cell line (Espevik *et al*., 1986). Cells were cultured in the presence of test samples (sera or culture supernatants) or recombinant murine TNF-α standards

(Genzyme, Cambridge, MA) ranging from 0.06 to 1000 pg/ml. After 20 hr, 10 µl/well of a 5 mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) were added for 4 hr. Then, the medium was decanted and the formazan product was dissolved with 100 µl/well of 95% ethanol for 10 min, with constant shaking. Absorbance was read at 578 nm on a Titertek Multiskan (Flow Laboratories, Finland). The lower detection limit was 0.1 pg/ml.

IL-6 was quantified by a proliferation assay using the murine B-cell hybridoma line B9 (Nelle *et al*., 1988). Cells were cultured in the presence of test samples (sera or culture supernatants) or recombinant murine IL-6 standards (AMS Biotechnology, Sweden) ranging from 0.12 to 8 pg/ml, during 72 hr. Then, 1 µCi/well of ^{3}H-thymidine (Amersham, UK) was added, and after 4 hr cells were harvested and thymidine incorporation was measured on a direct β-counter (Inotech, Switzerland).

IL-1α was quantified by a monoclonal antibody-based enzyme-immunoassay (Intertest 1α-X, Genzyme, Cambridge, MA) according to the manufacturer's instructions. The lower detection limit of the assay was 15 pg/ml.

Mitogenicity

The possible mitogenic action of *B. asper* venom on mouse lymphocytes was assessed by culturing spleen cells ($4x10^{5}$ cells/well; Bradley, 1980) in the presence of different venom concentrations, during 72 hr. Venom was sterilized by filtration through 0.22 µm membrane. As a positive control, *Escherichia coli* B 055:B5 lipopolysaccharide (10 µg/ml; Difco, Detroit, MI) was included. Cell proliferation was quantified by incorporation of ^{3}H-thymidine during 4 hr.

Cytotoxicity on mouse spleen cells

B. asper venom was added, in different concentrations, to spleen cells in 24-well plates ($2x10^{6}$ cells/well). After 6, 24, 48, and 72 hr, cells were harvested manually and the viability was estimated by an automated cell counter (Sysmex F-300, Toa Medical Electronics, Japan), in comparison to control wells in which venom was omitted.

Hematological alterations in vivo

B. asper venom (50 µg) was injected s.c. into the footpad of mice and at 0, 1, 3, 6, 12, 24, 72 hr, and 7 days, heparinized (200 U/ml) blood samples were obtained under anesthesia. White blood cells and platelets were counted in a Sysmex K-1000

analyzer (Toa Medical Electronics). Differential counts for leukocytes were performed on Giemsa-stained blood smears.

In vivo complement depletion

To assess the possible role of complement in the development of edema and IL-6 induction, Swiss mice were treated with an i.p. injection of "cobra venom factor" (Sigma) at a dose of 0.8 µg/g body weight, 16-18 hr before the respective experiments (Vogel, 1991). Control mice were treated with PBS. Complement depletion was verified by measuring serum C3 levels by radial immunodiffusion (Mancini *et al*., 1965), using a goat antiserum to mouse C3 (Cappel, North Carolina, U.S.A.) at a final concentration of 1% (v/v) in the agarose gel, and serum samples of 5 µl. The reduction in C3 levels obtained with this treatment was 91-93%.

Cytotoxicity on skeletal muscle myoblasts (L6) and endothelial cells (tEnd)

An *in vitro* quantitative assay for the cytotoxic activity of purified toxins was developed, using two cell lines: L6 rat skeletal muscle myoblasts (ATCC CRL 1458), and tEnd cells, a polyoma virus-transformed murine endothelial cell line of capillary origin (Bussolino *et al*., 1991), kindly provided by Dr M. Thelestam (Karolinska Institute, Sweden) and Dr A. Mantovani (Istituto di Ricerche Farmacologiche Mario Negri, Italy), respectively. Cells were routinely grown in Iscove's medium (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Biological Ind., Haemek, Israel), 2 mM L-glutamine, $5x10^{-5}$ M 2-mercaptoethanol, and 0.05 mg/mL gentamycin. In order to quantify cytotoxicity, cells were seeded in 96-well plates and grown for 2-4 days until near confluence. At the moment of the assay, culture medium was removed and replaced with 150 µl/well of medium with 1% FCS containing different amounts of toxins. The FCS concentration was lowered to 1% in order to minimize the basal lactate dehydrogenase (LDH; EC 1.1.1.27) activity of the medium. After 3 hr of incubation at 37°C (selected after time-course experiments), 100 µl of supernatant were assayed for LDH released from damaged cells (kit No.500, Sigma). As reference values for 100% and 0% cytotoxicity, cells were incubated with 0.1% Triton X-100-containing medium, or plain medium, respectively.

Cell surface heparan sulfate treatments

The possible role of cell surface heparan sulfate in the cytotoxic mechanism of

myotoxin II was investigated by (a) heparitinase digestion of cell cultures, (b) inhibition of heparan sulfate sulfation with sodium chlorate, and (c) the use of a cell mutant with a defect in heparan sulfate biosynthesis.

For heparitinase treatment, cell cultures were incubated with 1.25 U/well of heparinase III (Sigma), in 100 µl of PBS, for 2 hr at 37°C (WuDunn and Spear, 1989). Control cells were treated similarly but omitting the enzyme. Then, wells were washed twice with culture medium, and the cytotoxicity induced by myotoxin II was determined by LDH release. For chlorate treatment, cells were plated in microwells with medium containing 10 mM sodium chlorate (Greve *et al*., 1988; Olwin and Rapraeger, 1992) and grown for 2 days. At the moment of the assay, myotoxin II was added in chlorate-containing medium, and cytotoxicity was determined. Chlorate was omitted in control cultures. Finally, the cytotoxic action of myotoxin II was also quantified on two CHO cell lines, one defective in the synthesis of heparan sulfate (CHO-*pgs*D-677), and the wild type control (CHO-K1) (Lidholt *et al*., 1992), kindly provided by Dr. J.D. Esko (University of Alabama-Birmingham, U.S.A.).

Proteolytic and phospholipase A_2 activities

Proteolytic activity was tested by incubation of varying amounts of venom with 1% casein in PBS for 30 min at 37°C. Proteins were then precipitated by addition of trichloroacetic acid, and the increase of absorbance at 280 nm of the supernatants was determined (Lomonte and Gutiérrez, 1983). Phospholipase A_2 (PLA_2) activity of venoms or toxins was estimated by an indirect hemolytic assay in the presence of egg yolk phospholipids (Gutiérrez *et al*., 1988), either in gel or in fluid phase.

Fibrinolytic, coagulant, and anticoagulant activities

Fibrinolytic activity of venoms was determined on equine fibrin clots using the radial diffusion assay described by Gené *et al*. (1989). Coagulant activity was tested on human plasma at 37°C, by measuring the time to clot formation after adding varying amounts of venom, as described by Gené *et al*., (1989). Anticoagulant activity was determined as described by Alvarado and Gutiérrez (1988), by preincubating venom with platelet-poor human plasma at 37°C for 10 min, and then adding $CaCl_2$ to initiate coagulation.

Electrophoretic analyses and densitometry

Venoms, fractions, or purified toxins were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with or without reduction with 2-mercaptoethanol, on homogeneous 12 or 15% gels (Laemmli, 1970). Basic proteins were analyzed also by native cathodic PAGE on 12 or 15% gels (Reisfeld *et al*., 1962). All electrophoreses were run in a Mini-Protean cell (Bio-Rad, Richmond, CA, U.S.A.). Proteins were stained with Coomassie blue R-250 and, when pertinent, the gels were scanned on a GS300 Densitometer and analyzed with the GS365W Electrophoresis Data System (Hoefer Instruments, San Francisco, CA, U.S.A.) to determine the molecular weight and relative concentrations of the components.

Immunoblotting

Reactivity of the rabbit anti-*V. berus* serum towards the different venom components was tested by immunoblotting. Unreduced venom was separated by SDS-PAGE and transferred to nitrocellulose in a Mini-Transblot cell (Bio-Rad) (Towbin *et al*., 1979). Proteins on the nitrocellulose were reversibly-stained with amidoblack 10B (Syu and Kahan, 1987) to check for transfer efficiency. Strips were cut, blocked with bovine serum albumin and casein, and then incubated with either immune or normal rabbit sera. Bound antibodies were detected with an alkaline phosphatase anti-rabbit IgG conjugate, using nitroblue tetrazolium and 5-bromo-4-Cl-3-indolyl phosphate (Sigma) as substrates.

Gel diffusion

The interaction between heparins and *B. asper* venom or myotoxin II was tested by gel diffusion in 1% agarose-PBS plates (Ouchterlony and Nilsson, 1978). After 24 hr of incubation at room temperature, gels were washed with PBS and the precipitates stained with Coomassie blue R-250.

Enzyme-immunoassay

Detection of polyclonal and monoclonal antibodies to venoms or toxins was performed by direct enzyme-immunoassay on antigen-coated plates. After addition of samples, bound antibodies were detected by appropriate anti-Ig-enzyme conjugates (either with peroxidase or alkaline phosphatase) and subsequent color development, as previously described (Lomonte *et al*., 1991; Rovira *et al*., 1992). Normal sera from the same species was always used as a negative control.

A competitive enzyme-immunoassay was utilized to determine if heparin could inhibit the binding of MAb-3 (a neutralizing monoclonal antibody) to myotoxin II. Binding competition was assessed both by incubating a fixed amount of heparin with varying amounts of MAb-3, or a fixed amount of MAb-3 with varying amounts of heparin. Horse polyvalent antivenom was utilized as a positive control for competition in the binding of MAb-3 to myotoxin II.

Heparin-affinity chromatography

B. asper venom was fractionated on a column of heparin-agarose (Sigma) equilibrated with PBS, pH 7.2, in order to obtain the heparin-binding components. After the absorbance at 280 nm of the eluent returned to baseline, elution of the bound material was performed by either a stepwise or a linear gradient to 1 M NaCl. The salt-eluted fraction was then analyzed by SDS-PAGE and native cathodic PAGE as described above.

Heparin binding assay

A quantitative filter binding method (Maccarana *et al*., 1993), was utilized to study the interaction of myotoxin II with heparin, its derivatives, fragments, and other glycosaminoglycans (GAGs). The toxin was incubated at room temperature for 2 hr with the appropriate ^{3}H-labeled saccharide samples in 300 µl of 50 mM Tris-HCl, pH 7.4, 130 mM NaCl (TBS) containing 0.5 mg/ml of bovine serum albumin. The protein, along with any bound carbohydrate, was recovered by quick passage of the mixtures through nitrocellulose filters (Sartorius, pore size 0.45 µm; 25 mm diameter) which had been placed onto a 10-well vacuum-assisted manifold filtration apparatus. The filters were prewashed twice with 5 ml of TBS, before application of the samples, which were immediately followed by another two washings with the same buffer; each washing step was completed within 5 sec. Protein-bound radioactivity was determined after submersion of the filters in 2 ml of 2 M NaCl for 30 min; 1.5 ml of the eluate was mixed with 1 ml of distilled water and 8 ml of scintillation cocktail (OptiPhase, Pharmacia LKB Biotechnology, Sweden) and counted in a Beckman LS 6000IC scintillation spectrometer. No residual radioactivity could be detected on the filters.

RESULTS

Inflammatory response to Bothrops asper snake venom (paper I)

B. asper venom induced a potent inflammatory response in the mouse when injected s.c. in the footpad. Both local and systemic alterations were observed. Locally, a striking immediate edema was elicited, which lasted from 12 hr to more than three days, depending on the dose injected. The edema could be induced in the absence of a hemorrhagic effect, although bleedings were associated with a more prolonged edema. A diffuse inflammatory infiltrate accumulated by 6 hr in the injected footpad, both in the subcutaneous and muscular tissues (showing hemorrhage and myonecrosis), predominantly consisting of polymorphonuclear neutrophils. At 24 and 72 hr the cellular infiltrate increased and mononuclear phagocytes appeared, in addition to the neutrophils. After one week the infiltrate had almost completely disappeared, except for some fibrotic foci still containing macrophages.

Systemically, significant hematological alterations were induced by the venom. Platelets decreased almost immediately and did not normalize until 12 hr after venom injection. A moderate leukocytosis and lymphopenia was observed, with a rapid inversion of the ratio between these two cell types during the first 6 hr, which returned to normal by 24 hr.

A rapid elevation of serum IL-6 levels was induced by the venom, peaking at 3-6 hr and returning to normal by 12 hr. No elevations of TNF-α and IL-1α could be detected in serum after venom injection. This was not due to the detection systems, since control animals injected with *E. coli* LPS had high serum levels of both cytokines. Additional control tests indicated that the venom, at the concentrations utilized, did not interfere with the indicator systems for cytokine quantification, neither changed the activity of recombinant cytokines after their preincubation.

The systemic IL-6 response was obtained also after s.c. injection of purified myotoxin II, or of whole venom previously incubated with an amount of antivenom that completely neutralized its hemorrhagic activity (paper I). Additional studies showed that a high IL-6 response could be similarly induced by two other venoms tested (*Vipera berus* and *Crotalus viridis viridis*) and, as well, by the purified hemorrhagic toxin BaH-1 from *B. asper* venom (Table 2).

IL-6 production could not be induced by directly incubating the venom with mouse spleen cell suspensions *in vitro*, while a moderate dose-dependent cytotoxic action was detected. The venom was not mitogenic for splenocytes either.

An experiment performed to explore the possible involvement of endothelial cells in the production of IL-6 in response to venom or myotoxin II showed a slight, but significant ($p<0.01$), increment of IL-6 released to the supernatant, at 9 and 24 hr (Fig.1). The i.v. injection of human recombinant IL-6 (100 ng) into mice, 6 hr before the i.d. injection of *B. asper* venom, did not result in a significant decrease of the hemorrhagic effect in comparison to untreated controls (Table 3). In addition, preincubation of *B. asper* venom with human α_2-macroglobulin (α_2M), at a ratio of 50 µg venom/mg α_2M, did not reduce its hemorrhagic activity in mice ($p>0.05$, $n=4$).

Table 2: Serum IL-6 induction 3 hr after the s.c. footpad injection of snake venoms or hemorrhagic toxin BaH-1, in mice.

Substance	Dose	n	IL-6 concentration (pg/ml)*
Vipera berus venom	50 µg	4	2374 ± 825
Crotalus viridis viridis venom	50 µg	3	5205 ± 1215
BaH-1 hemorrhagic toxin	5 µg	3	1684 ± 384

*Serum IL-6 levels in normal mice were < 20 pg/ml.

Table 3: Effect of the preinjection of recombinant human IL-6 on the hemorrhagic activity of *Bothrops asper* venom, in mice*.

Group	n	Hemorrhagic area (mm^2)	
Control	5	83 ± 29	
rhIL-6 treated	5	65 ± 27	N.S.

*Control mice were left untreated, while the experimental group received 100 ng of recombinant human IL-6 i.v., 6 hr before the assay of hemorrhage induced by 10 µg of venom administered i.d. N.S.: non significant difference ($p>0.05$).

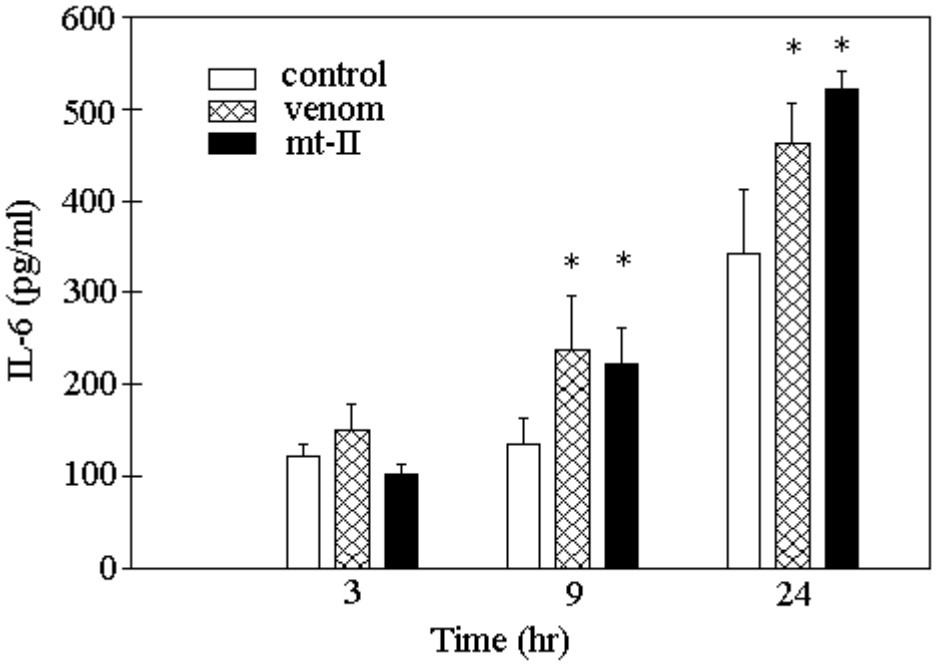

Figure 1: IL-6 release from endothelial cells (tEnd) cultured in the presence of medium only (control), *B. asper* venom (0.1 µg/ml), or myotoxin II (5 µg/ml). Each bar represents mean ± SD of four independent cultures, each supernatant assayed in triplicate. The asterisks indicate a statistically significant ($p<0.01$) difference of the values in comparison to the control values at the same time point.

The possible involvement of complement in the mechanism of induction of the IL-6 response and edema by *B. asper* myotoxin II, was explored by pretreating mice with "cobra venom factor". This treatment resulted in a 91-93% reduction of serum C3. As shown in Fig.2, no difference with the control group could be observed in the complement-depleted mice regarding footpad edema. Similarly, complement depletion did not modify significantly the IL-6 response elicited by i.m. myotoxin II injection (Table 4).

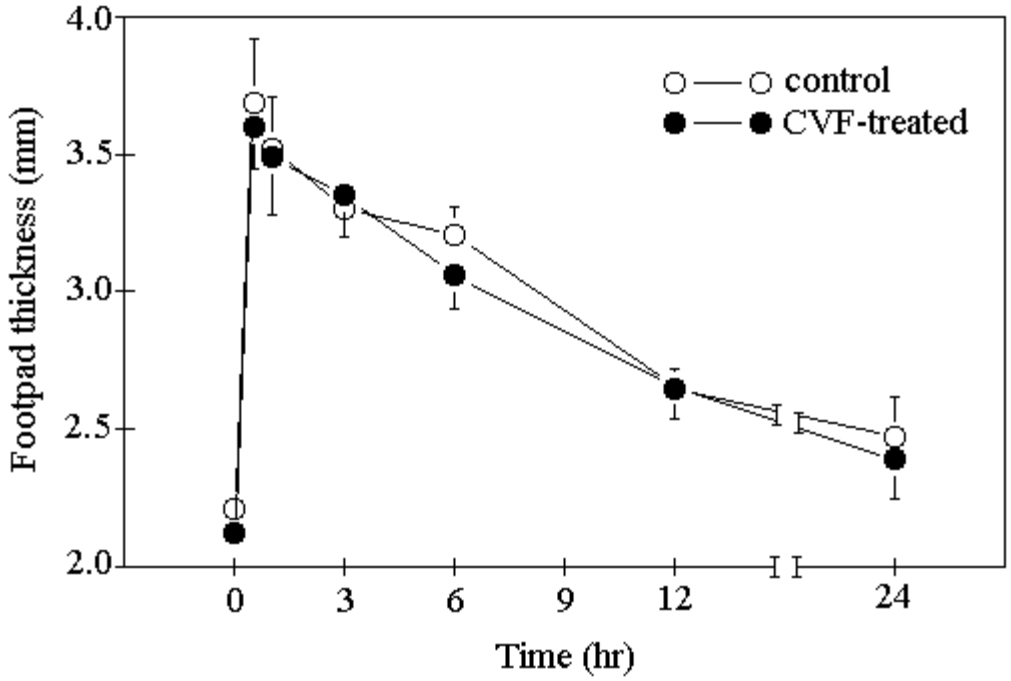

Figure 2: *In vivo* complement depletion by "cobra venom factor" (CVF) pretreatment does not affect the footpad edema induced by myotoxin II in mice. CVF (or PBS for the control group) was given i.p. (0.8 µg/g body weight) 18 hr before toxin injection (100 µg). Each point represents mean ± SD of four mice.

Table 4: Serum IL-6 response elicited by i.m. injection of *B. asper* myotoxin II in mice pretreated with "cobra venom factor (CVF)"*.

Group	n	Serum IL-6 (pg/ml)
(1) Myotoxin II	3	1510 ± 240
(2) CVF + Myotoxin II	3	1711 ± 450
(3) CVF only	3	138 ± 46

*CVF i.p. treatment was 0.8 µg/g body weight, 18 hr before the i.m. injection of myotoxin II [100 µg, (1) and (2)], or PBS (3). IL-6 was measured 3 hr after toxin injection. The difference between (1) and (2) is not significant, while group (3) is moderately increased in comparison to normal levels (<20 pg/ml).

Characterization of the venom of Vipera berus (paper II)

V. berus venom was highly lethal to mice (i.p. LD_{50} of 0.86 ± 0.15 µg/g body weight) and induced significant local tissue damage characterized by marked hemorrhagic and edema-forming effects, and a moderate myotoxicity, in the mouse. The footpad edema was rapid, drastic, and its duration was related to the dose injected. The minimum hemorrhagic dose obtained was 3.2 µg. Local myotoxic activity was demonstrated by histological evaluation 24 hr after i.m. injection, and by the dose-dependent creatine kinase release to plasma, measured at 3 hr, or by the decreased muscle MTT-reducing activity measured at 24 hr. *In vitro*, this venom had a moderate proteolytic activity on casein (59.2 U/mg) and displayed PLA_2 activity, detectable by an indirect hemolytic assay in gel with 0.3 µg venom/well or higher amounts. Fibrinolytic and anticoagulant activities were low, and there was no coagulant effect on human plasma.

An antiserum to *V. berus* venom showed significant cross-reactivities to several crotalid venoms by enzyme-immunoassay, ranging from 62% for *Bothrops asper* to 18% with *Crotalus durissus durissus*. *V. berus* venom was resolved into 10-11 protein bands when electrophoretically separated on the basis of size by SDS-PAGE. All the bands were recognized by the rabbit antiserum in immunoblots from unreduced SDS-PAGE separations.

New method for quantification of myonecrosis (paper III)

The venom of *Micrurus nigrocinctus* was utilized in the development of a method for the estimation of myonecrosis in mice, and evaluation of the neutralizing ability of antibodies. Normal muscle (gastrocnemius) homogenates prepared in the presence of 1% Triton X-100 were found to contain MTT-reducing activity. The purple formazan product remained in solution, eliminating the need for further solubilization procedures. The muscle MTT-reducing activity decreased rapidly after venom injection, reaching a plateau at about 20% residual activity after 24 hr. This time point was selected for all subsequent experiments. The evaluation of dose-response curves for venom-induced myonecrosis, in the range of 0.6-20 µg/muscle, showed that the residual MTT-reducing activity correlated well with the number of viable cells in the tissue, as determined by quantitative histological evaluation. This correlation was better than that observed when measuring the residual CK activity of the muscle, which overestimated myonecrosis in comparison to the histological evaluation.

In plasma, a dose-dependent increase in CK levels was observed at 3 hr, over the whole range tested, still increasing at doses which had destroyed virtually all muscle fibers in the injected gastrocnemius, as determined by the histological reference technique.

The use of the MTT-based technique in asessing the neutralization of myotoxic activity of *M. nigrocinctus* venom by an antivenom was evaluated. The method indicated a complete inhibition of venom myotoxins at an antivenom/venom ratio of 4 ml/mg or higher, with a 50% effective dose (ED_{50}) of approximately 2.5 ml/mg.

Dynamics of the local tissue damage induced by Bothrops asper venom (paper IV)

The acute tissue damaging effects of *B. asper* snake venom and myotoxin II on the mouse cremaster muscle, were studied by intravital and electron microscopy. Muscle fibre contractions appeared between 10 and 60 sec after contact between venom and tissue, and caused a pronounced mechanical distortion of the microvasculature. At the same time, sphincter-like arteriolar constrictions were observed, causing intermittent reduction in the blood flow in the depending capillary networks for about 1 min. Both muscle contractions and arteriolar constrictions ceased within 2 min, and did not reappear even when the muscle was exposed to venom for up to 30 min. Another early event, occurring within 1 min and continuing during the whole observation period, was the formation of thrombi in the postcapillary venules, giving rise to numerous emboli, and an accumulation of leukocytes along the venular endothelium.

Leakage of i.v. injected FITC-dextran started about 2 min after venom exposure, and seemed to originate mainly from small venules and their adjoining capillary segments. About 5-6 min after the application of the venom, a large proportion of the observed area showed diffuse leakage. Stasis in the microcirculation, with a dense packing of erythrocytes in capillaries and small venules occurred at about the same time as the plasma leakage.

Microbleedings regularly appeared after about 4-6 min of exposure and were always explosive in character. They originated from capillaries and small venules, and often developed as a series of burst-like enlargements of the microhematoma before stopping within about 10-20 sec. In many cases the blood flow through a ruptured microvessel continued after the bleeding had stopped. No bleedings originated from arterioles. Electron microscopy (EM) revealed that seemingly intact and ruptured microvessels could be found adjacent to each other in a damaged tissue area. Furthermore, the EM showed that erythrocytes escaped through gaps in the

endothelial wall, with indications that some cellular component was fragmented at that site. Another striking phenomenon in the microcirculation, noted both intravitally and by EM, was the accumulation of spherical, but apparently not hemolysed, erythrocytes.

As dramatic in appearance as the bleedings, but often starting already after about 3-4 min, was the degeneration of muscle fibers. The process started as a focal loss of striation seen as a narrow band across the entire width of the fiber, followed by the appearance of a small, wedge-shaped lesion at one edge of the fiber and a slow retraction of the myofibrils in opposite directions, which widened the gap until the fiber suddenly ruptured. This rupturing was often repeated along the same fiber so that in the end there was only a row of fragments separated by spaces, apparently devoid of myofibrillar material. Despite this severe morphological alteration of the tissue, there was still blood flow in apparently undamaged microvessels running along and across the affected muscle fibers.

Application of myotoxin II to the cremaster resulted in a similar, rapid reaction as with venom, i.e. there were muscle contractions and a concomitant distortion of the microvasculature, but there were no arteriolar constrictions. The microvascular permeability also increased, although to a lesser extent than with whole venom. No thrombi, emboli or bleedings were observed during the experiments. The main lasting effect was the drastic alteration of muscle fibers, which was, dynamically and morphologically, indistinguishable from that caused by whole venom.

Effects of hemorrhagic toxin BaH-1 on endothelial cells (paper V)

The effects of BaH-1, a hemorrhagic metalloproteinase from *B. asper* venom, on cultured endothelial cells, were studied. The toxin, despite its potent hemorrhagic action *in vivo*, did not induce any significant cytotoxicity on the capillary endothelial cells *in vitro*, even when concentrations as high as 65 µg/ml were tested. The toxin *per se* did not interfere with the LDH assay utilized for the assessment of cell damage. The main visible effect of toxin BaH-1 on endothelial cells was a relatively slow, moderate detachment, with some of the cells becoming round. This effect was clearly dose-dependent, but did not cause any intracellular LDH release to the medium. In contrast, myotoxin II, which is devoid of hemorrhagic activity, was cytotoxic to the endothelial cells, killing 100% of the cells under similar assay conditions.

Neutralization of Bothrops asper myotoxins by heparins (paper VI)

When whole *B. asper* venom was preincubated with either standard or LA-heparin, and subsequently injected into mice, its myotoxic action was significantly reduced in a dose-dependent manner. This result was confirmed by histological evaluation and by the use of intravital microscopy. In the latter system, widespread muscle fiber damage regularly developed 4-6 min after application of venom alone, whereas, when it was mixed with standard or LA-heparin before application, muscle fibers were protected throughout the observation period of 30 min. In contrast to LA-heparin, standard heparin markedly increased the hemorrhage induced by the venom, evident both histologically and intravitally.

The venoms of *B. jararacussu*, *Agkistrodon p. piscivorus* and *Trimeresurus flavoviridis*, were also significantly neutralized by LA-heparin, regarding myotoxic activity, as judged by the reduction in the serum CK levels measured at 3 hr. However, the venoms of *Crotalus durissus terrificus*, *C. viridis viridis*, *Naja naja atra*, and *Vipera berus*, were not neutralized.

Myotoxin II was cytolytic to L6 myoblasts and tEnd endothelial cells, in the concentration range of 25-150 µg/ml. Endothelial cells were significantly more susceptible than myoblasts to this cytotoxic action. Morphologically, both cell types showed, after exposure to the toxin, an abundant cytoplasmic granulation, followed by an apparent dissolution of the membrane, without detachment, similar to the appearance of detergent-treated cultures.

When preincubated with myotoxin II, standard as well as LA-heparin blocked its cytolytic activity on both cell types. Cells were completely protected at approximate ratios of 0.02 and 0.3 µg heparin/µg myotoxin II, for L6 and tEnd cells, respectively, with no observable differences in the inhibitory efficiency of the two heparin types. Since the average molecular weight of both heparins utilized (15 kD) is similar to that of myotoxin II, the heparin/toxin ratios expressed as µg/µg roughly correspond to molar ratios. Under identical conditions, the neutralizing monoclonal antibody MAb-3 was also able to inhibit the cytolytic action of myotoxin II, completely preventing its effect at an approximate molar ratio of 1:1.

A complete neutralization of cytolysis caused by myotoxin II was also achieved with heparan sulfate and low molecular weight heparin (Fragmin®), although not with a variety of heparin-derived disaccharides or with chondroitin sulfate. A non-anticoagulant heparin preparation of low molecular weight (LMW-LA-heparin) also inhibited myotoxin II *in vitro*, although with a slight reduction in

neutralization potency (Fig.3). On the other hand, pretreatment of the cells with heparins, followed by washing, had no protective effect on myotoxin II-induced cytolysis.

A significant inhibition of edema in the mouse footpad assay was achieved by preincubation of myotoxin II with LA-heparin .

The heparin-binding components in *B. asper* venom were isolated by affinity chromatography, eluting at approximately 0.5 M NaCl. SDS-PAGE of this fraction showed a main band of 14-15 kD under reducing conditions, which corresponds to the subunit molecular weight of myotoxins, and very small amounts of few other components. Native cathodic PAGE showed that all described myotoxin isoforms bound to the immobilized heparin column. Gel diffusion demonstrated that heparins form a precipitable complex with venom myotoxins.

Since affinity chromatography and electrophoresis showed that heparin interacts not only with myotoxin II, but also with other isoforms present in this venom, the effect of heparins on the enzymatic and myotoxic activities of myotoxin III was investigated. Both types of heparins significantly reduced the myotoxic effect of this isoform *in vivo*, without inhibiting its enzymatic activity in an indirect hemolysis assay.

The possible role of cell surface heparan sulfate in the mechanism of cytotoxicity induced by myotoxin II was investigated. Both heparitinase and chlorate treated L6 and tEnd cells were equally susceptible to myotoxin II, in comparison with their respective controls. In agreement with this, myotoxin II induced a comparable cytolytic effect in heparan sulfate defective mutant cells CHO-*pgs*D-677 and CHO-K1 control cells.

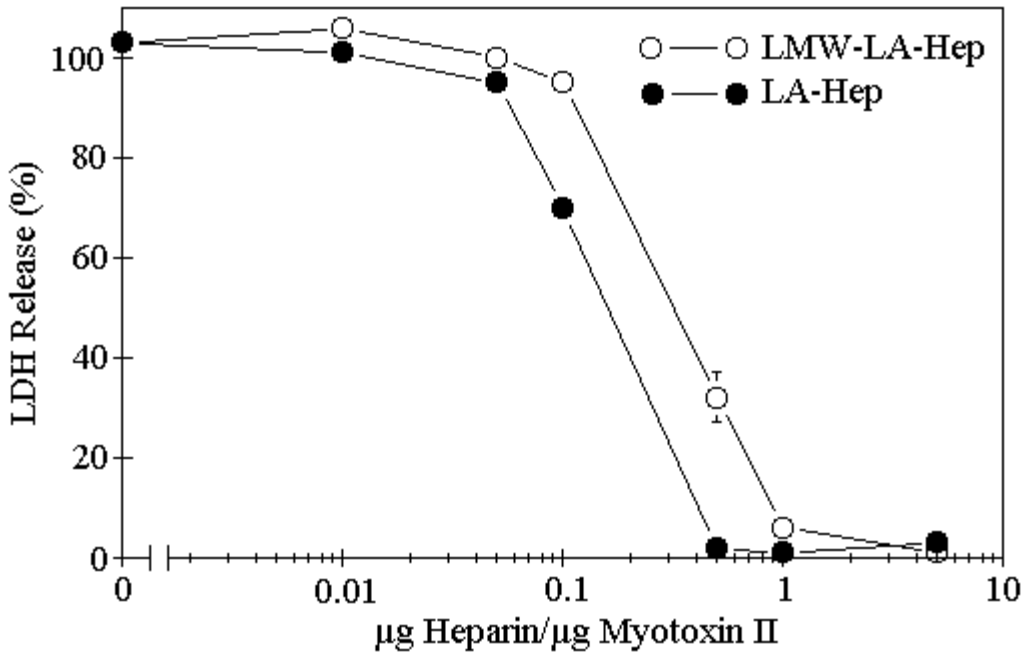

Figure 3: Neutralization of the cytolytic effect of *B. asper* myotoxin II on cultured endothelial cells (tEnd) by preincubation with non-anticoagulant heparin preparations of different molecular weight: LA-heparin ~15 kD (filled circles), and low molecular weight-LA-heparin ~4 kD (open circles). Each point represents mean ± SD of duplicate assays. LDH: lactic dehydrogenase.

Structural characteristics of the heparin-myotoxin interaction (paper VII)

The direct binding of ^{3}H-labeled heparin to myotoxin II was measured by a nitrocellulose filter binding assay. Myotoxin II bound almost all of the added heparin in solution, in an unselective manner. The ability of different unlabeled glycosaminoglycans (GAGs) to interact with myotoxin II was studied. When the ability to compete with ^{3}H-labeled heparin was tested, none of the GAGs was a better competitor than heparin. Selective O-desulfation of heparin resulted in a moderate decrease in the interaction, approximately two-fold for 6-O-desulfated heparin, and six-fold in the case of 2-O-desulfated heparin. The interaction was even weaker with the heparin desulfated at both 2-O and 6-O positions. Other sulfated GAGs, such as dermatan sulfate and chondroitin sulfate, competed poorly in the binding, only at concentrations about two orders of magnitude higher than that of heparin.

Neutralization of the cytolytic activity of myotoxin II was in agreement with both direct and inhibition binding data. Titration of the unmodified heparin indicated a complete neutralization of toxin activity at the ratio of 1 µg saccharide/µg protein or higher. Both 2-O- and 6-O-desulfated heparins were able to neutralize the toxin at a ratio of 1 µg/µg, while no inhibitory effect could be obtained with chondroitin sulfate or a low-sulfated heparan sulfate preparation, even at ratios ten times higher than those required in the case of heparins.

The minimal size of heparin required to interact with myotoxin II was determined both by direct binding of ^{3}H-labeled oligosaccharides and by neutralization of the cytolytic activity. The shortest heparin-derived oligosaccharides interacting with the protein were hexasaccharides. The level of binding increased proportionally to the saccharide length. In agreement with these data, when unlabeled heparin-derived oligosaccharides were tested as myotoxin-neutralizing compounds, at a ratio of 1 µg/µg of protein, hexasaccharides gave a partial protective effect, octasaccharides showed an almost complete protection, and decasaccharides or larger species blocked completely the cell-damaging activity of the toxin.

Macromolecular complex formation was investigated by adding increasing amounts of heparin to a myotoxin II solution, and then measuring the turbidity at 340 nm. Myotoxin II alone did not absorb light at this wavelenght. A maximal turbidity was obtained at a ratio of 0.2 µg heparin/µg protein (5 protein molecules/heparin chain), being constantly diminished by further additions of heparin.

Since both heparin and the monoclonal antibody MAb-3 neutralize the cytotoxic activity of myotoxin II, the possible competition between these two molecules for toxin binding was investigated using an enzyme-immunoassay. Results showed that heparin, even at very high concentrations, was not able to inhibit the binding of MAb-3 to myotoxin II. As a control, a polyclonal antivenom preparation competed with MAb-3 in a dose-dependent mode.

A synthetic peptide corresponding to the N-terminal 26 amino acids (described as a heparin-binding site of porcine pancreatic PLA_2) of myotoxin II was tested for its ability to bind heparin. No significant heparin binding was observed in the filter assay, despite that the peptide could bind to the nitrocellulose filter. Moreover, in the cytotoxicity assay, the presence of peptide (up to 275 µg/ml) did not affect the neutralizing activity of heparin towards myotoxin II. The peptide itself did not have any cytotoxic effects even at these high concentrations.

GENERAL DISCUSSION

The purpose of the present work was to characterize the local tissue damage and inflammatory reactions induced by snake venoms and toxins, to develop new methods for the study of tissue-damaging venom components, and, finally, to find and characterize potentially neutralizing agents to these toxins.

Inflammation is a major feature of envenomations by many snake species, yet its possible contribution in the pathogenesis of venom effects, or, on the other hand, its possible antagonisms to toxic actions, have received relatively little attention. One reason for this has probably been the intrinsic complexity of inflammation as an integrated physiological process. Nevertheless, a precise understanding of the inflammatory events that follow envenomation, and their roles, could lead to the development of better therapeutic strategies, complementary to conventional serotherapy (Hanson, 1991).

We investigated the host response to *Bothrops asper* venom in a mouse model, with particular emphasis on edema, leukocyte infiltration, and the possible participation of cytokines as mediators of this inflammatory process (paper I).

Two types of edema responses to the venom were observed. At low doses (i.e. 1 µg), a rapid but transient edema developed, in the absence of hemorrhage, and with a scarce leukocyte infiltrate in the injected footpad. At this low dose, probably only the true permeability-increasing components of the venom are being detected, in the absence of additional contributing factors. However, at higher venom doses (i.e. 50 µg), more representative of the clinical and pathological picture observed after snakebites, there was a similarly rapid edema, but it was sustained over a much longer period. In this case, the edema was accompanied by hemorrhage, myonecrosis, and abundant leukocyte infiltration. The duration of the edema was related to the dose of venom injected (paper I). A similar finding was made in the study of the edema induced by the venom of *Vipera berus* (paper II). Although both observations strictly correspond to an animal model, they support the concept that the magnitude and duration of local edema could be simple and useful parameters for the estimation of severity of envenomation by crotalids in humans.

Leukocytes infiltrated the tissues within 3-6 hr after venom injection, the predominant type being the neutrophil (paper I). This infiltrate peaked at 24 and 72 hr and declined thereafter. Mononuclear phagocytes were also observed at these later times, but relatively few lymphocytes appeared in the inflamed footpad tissues. The scarcity of infiltrating lymphocytes is in agreement with the report of Emslie-

Smith and Harris (1989) in the inflammatory response induced by notechis II-5, a myotoxic PLA_2 of *Notechis scutatus* venom, in the rat. In our studies, the appearance of a cell infiltrate was independent of the hemorrhagic action of the venom, since it could be observed after injection of (a) venom that was partially preneutralized with antivenom, in the absence of hemorrhage, and (b) purified myotoxin II. This suggests that myonecrosis is, at least in part, responsible for the extravasation of neutrophils and mononuclear phagocytes into the tissues. The kinetics and cell composition of this inflammatory response appeared similar to that previously described after the injection of *N. scutatus* (Harris and Johnson, 1978) and *B. asper* (Gutiérrez *et al*., 1986*f*) venoms into skeletal muscle. Phagocytes have been commonly found in areas of myonecrosis induced by venoms or toxins, and are important in the removal of dead cells and debris in the process of muscle regeneration (Harris *et al*., 1975; Queiroz *et al*., 1984; Kouyoumdjian *et al*., 1986; Ownby, 1990; Harris and Cullen, 1990; Gutiérrez *et al*., 1984*c*, 1986*d*, 1990*b*; Sharp *et al*., 1993). Moreover, phagocyte-derived enzymes probably participate in the degradation of myofibrillar proteins after myonecrosis (Pluskal *et al*., 1978; Gutiérrez *et al*., 1990*b*).

Several hematological alterations occurred after venom injection (paper I). Circulating platelets abruptly decreased, and did not normalize until 12 hr later. This finding is in agreement with intravital microscopic observations of platelet thrombi forming on venular walls shortly after exposure to venom (paper IV). Thrombocytopenia can frequently occur in human envenomations by snakes (Russell *et al*., 1975; Warrell *et al*., 1976*b*; Reid and Theakston, 1983; Kamiguti *et al*., 1991*b*, 1992; Benbassat and Shalev, 1993; Cardoso *et al*., 1993). In a clinical study of eighteen patients bitten by *B. asper*, no significant changes in platelet counts were found, despite the marked alterations in coagulation parameters (Barrantes *et al*., 1985). Although species differences cannot be ruled out, the discrepancy with our findings may be due to the time in which samples were analyzed: in the patients, tests were performed between 2 and 24 hr after the snakebite, and, at least in our mouse model, platelet numbers had already normalized 12 hr after venom injection. A similar reason may also explain the findings in a case of juvenile *B. asper* bite, in which the dramatic coagulation alterations were not accompanied by a decrease in platelet numbers: the patient was first evaluated 20 hr after the accident (Kornalík and Vorlová, 1990).

In the case of *B. asper* venom, it remains to be determined which component(s) is(are) responsible for the thrombocytopenic effect. Hemorrhagic toxins, by inducing

important endothelial cell damage *in vivo* (Ownby *et al*., 1978, 1990; Ownby and Geren, 1987; Kamiguti *et al*., 1991*a*; Moreira *et al*., 1994) would be likely candidates. A hemorrhagic fraction isolated from *B. jararaca* venom was capable of inducing a marked thrombocytopenia in rats after 2 hr (Kamiguti *et al*., 1991*a*), similar to the effects described in our experiments with crude *B. asper* venom. Unfortunately, the small amounts of purified hemorrhagic toxin BaH-1 available (paper V) precluded our possibilities to evaluate this hypothesis by intravital and hematological analyses.

Regarding cytokine involvement in venom-induced inflammation, a significant increase of serum IL-6 concentration was observed soon after injection, while no IL-1α and TNF-α increases were detected (paper I). Preliminary immunohistochemical data indicate, however, that some local IL-1α production in response to envenomation may occur (unpublished data). This cytokine response pattern is similar to that observed in acute trauma conditions (Cruickshank *et al*., 1990; Hoch *et al*., 1993) and thus, might be an indirect response to the tissue damage caused by various venom components. Indeed, the IL-6 serum peak was induced not only by the whole venom, but also by the injection of (a) isolated myotoxin II or (b) hemorrhagic toxin BaH-1, suggesting that myonecrosis and endothelial cell damage could both be initial stimuli for the IL-6 response. These observations, together with the inability of venom to stimulate IL-6 release from splenocytes (containing macrophages, T and B lymphocytes) *in vitro*, strongly suggest that the IL-6 production is triggered in response to cell damage. Other snake venoms tested, such as *Vipera berus* and *Crotalus v. viridis*, also elicited high IL-6 responses, showing that this phenomenon is not unique to *B. asper* venom, but probably a common feature of tissue-damaging venoms.

The cell type responsible for IL-6 release *in vivo* in the present model, as well as the signaling mechanisms involved, are not known. Indeed, surprisingly little is known about the primary signals that trigger IL-6, and the subsequent acute-phase response, in non-infectious conditions involving tissue damage (Koj *et al*., 1993). Our pilot *in vivo* experiments exploring the possible role of complement in the signaling for IL-6 release, after muscle necrosis induced by myotoxin II, were negative. *In vitro*, the small, but significant increase in IL-6 release by endothelial cells treated with *B. asper* venom and myotoxin II, could be suggestive of a role of this cell type *in vivo*. However, these results must be interpreted with caution. Although both agents were tested at subcytolytic doses, further experiments showed that myotoxin II exerts membrane-permeabilizing effects on this and other cell types

(papers V and VI), and thus, the small increase of IL-6 in culture supernatants may well be the result of a minor, undetected, cell membrane damage, rather than a direct induction of IL-6 synthesis and secretion. Analyses at the level of IL-6 mRNA would be necessary to discern between the two possibilities.

To our knowledge, this is the first report dealing with the evaluation of proinflammatory cytokines in the response to snake venoms. Our findings in the mouse model are in agreement with the results recently obtained in a collaborative clinical study of snakebites by *Bothrops* spp. and *Crotalus durissus terrificus* from Brazil (Barraviera, Lomonte, Tarkowski, Hanson, and Meira, manuscript submitted). In the sera of these patients, IL-6 elevations were frequently observed , together with IL-8 (not analyzed in the present work), while some IL-1β and no TNF-α increases could be detected. An increase in IL-6 levels could explain the transient lymphopenia and leukocytosis observed both in mice (paper I) and in these snakebite patients, since this cytokine is capable of increasing adrenocorticotropic hormone and glucocorticoid secretion (Castell *et al*., 1989). Lymphopenia has also been described after injection of notechis II-5 from *Nothechis scutatus* venom (Emslie-Smith and Harris, 1989). Our observations on the rapid IL-6 response induced after venom or myotoxin II-induced myonecrosis may explain the findings of these authors.

IL-6 is known to be an important signal for inducing the synthesis of acute-phase proteins in the liver (Mackiewicz *et al*., 1988; Castell *et al*., 1989; Lewis *et al*., 1992). Several of its actions might be beneficial in the pathological situation induced by snake venoms. Speculatively, the increased anti-protease production by hepatocytes might inhibit some venom proteases, as well as endogenous enzymes activated and released during inflammation. Although we did not observe a neutralizing effect of purified human α_2-macroglobulin when preincubated with crude *B. asper* venom, the interaction of this proteinase inhibitor with pure hemorrhagic toxins from *Crotalus atrox* venom has been demonstrated (Baramova *et al*., 1990*b*). Similarly, fibrinogen synthesis increase may compensate the fibrinogen consumption caused by the venom (Barrantes *et al*., 1985; Gené *et al*., 1989). IL-6 can increase platelet numbers (Hirano *et al*., 1990), and thus, may be responsible for the observed normalization of this parameter at 12 hr, just after the cytokine peak (paper I). IL-6 can stimulate the division of myoblasts *in vitro* (Austin and Burgess, 1991), and thereby could have a role in the process of regeneration of skeletal muscle after necrosis. IL-6 is known to induce renal mesangial cell proliferation (Roitt *et al*., 1993), a pathological finding that has been described after the injection

of *Trimeresurus flavoviridis* (Barnes and Abboud, 1993) and *Bothrops jararaca* (Rezende *et al*., 1989) venoms. However, all these possible actions of IL-6 are theoretical. We attempted to detect a possible induction of acute-phase reactants with anti-hemorrhagic activity in the mouse, by IL-6 injection and subsequent quantitative assay of hemorrhage induced by *B. asper* venom. However, this pilot experiment failed to show any reduced hemorrhage in mice receiving the cytokine 6 hr before the venom, in comparison to untreated controls. Although the recombinant IL-6 utilized was of human origin, it is known to be active on murine cells (Van Snick, 1990). An extremely valuable tool for future studies of the possible roles of this cytokine in envenomation models, will be the recently developed mouse strain with a targeted IL-6 gene deletion (IL-6 "knock-out" mouse).

As in other models of tissue damage and inflammation, these two processes are intimately related, making it difficult to determine the extent to which inflammatory reactions are causes or effects of damage, or the delineation between detrimental or beneficial consequences to the host. At least one possible cytokine with known tissue damaging ability, TNF-α (Dinarello, 1992), could not be demonstrated in this model. However, still many potent inflammatory mediators with destructive abilities remain to be investigated in relation to the local tissue damage caused by venoms.

A tentative scheme summarizing some of the events and interactions during local tissue damage and inflammatory responses induced by snake venoms, is depicted in Fig. 4. This general scheme constitutes only a personal view of the process, and it should be emphasized that is partly hypothetical, as some of the possible interactions and effects have not been specifically documented, and important variations are expected depending on different venoms, etc.

A powerful tool in the study of acute local tissue damage and inflammatory reactions induced by venoms, as well as in many other pathological entities, is the use of intravital microscopy techniques (Menger and Lehr, 1993). The effects induced by particular isolated toxins can be compared to those caused by the whole venom, in order to dissect the complex series of reactions occurring *in vivo*. In this investigation, the characteristics of the early pathological alterations caused by *B. asper* venom and myotoxin II were microscopically analyzed in the cremaster muscle of live mice (paper IV). The immediate muscle fiber contractions occurring after contact with venom or myotoxin II, are in agreement with the notion that myotoxins act initially on the sarcolemma, altering its permeability and allowing a calcium influx. However, other possible causes for this effect cannot be ruled out. Blood flow disturbances were evident in the tissue microcirculation, and were

caused by a combination of factors, including: (a) thrombotic activity on the walls of venules (but not arterioles); (b) marked stasis and hemoconcentration, concomitant to the abundant plasma leakage visualized by FITC-dextran extravasation; (c) shape changes of the erythrocytes such as crenation and sphering, altering their rheological properties; and (d) microvessel damage and hemorrhage. Interestingly, however, in many cases the blood flow through a ruptured microvessel continued after the bleeding had stopped (in about 10-20 sec). All these mechanisms affecting blood flow probably play a role in the development of ischemic conditions leading to further, secondary muscle necrosis of slower onset, in addition to the direct and rapid muscle fiber damage induced by venom myotoxins.

The early morphological manifestations of the process of muscle fiber damage were observed (paper IV), and were similar in the case of whole venom and purified myotoxin II. The degeneration of individual fibers appeared initially as a focal loss of striation, seen as a narrow band across the entire fiber width, and was followed by the appearance of a small wedge-shaped lesion at one edge, retraction of the myofibrils in opposite directions, and widening of the gap until a complete rupture had occurred. The process could be gradually repeated at several sites along the same fiber, leaving in the end a row of amorphous fragments separated by apparently empty spaces. Interestingly, at a relative short distance from cellular rupture sites, normal striations were still visible, until the process also spread into these areas. Therefore, muscle fiber damage caused by myotoxin II or venom, does not develop as a homogeneous degeneration of the whole fiber or large parts of it, but has a markedly focal character. Again, this observation is in agreement with the notion of a rapid, membrane-acting mechanism of myotoxins.

Also intravitally, it was possible to determine that the main targets of the hemorrhagic process caused by the venom are microvessels, in the size range of capillaries and small venules (paper IV). The explosive character of the erythrocyte extravasation process is in agreement with the concept of hemorrhage *per rhexis*. This was also supported by electron microscopic observations of the affected vessels, showing gaps in the endothelial wall, through which erythrocytes escaped.

The *in vivo* mechanism of action of hemorrhagic toxins has not been completely established. While considerable evidence points out to a mechanism based on the proteolytic degradation of the basal lamina of microvessels as the key event, notorious degenerative changes in endothelial cells also occur, and therefore the possibility that hemorrhagic toxins might have direct damaging effects on this cell type has been speculatively suggested (Ownby and Geren, 1978; Ownby *et al.*,

1990; Gutiérrez and Lomonte, 1989). Our *in vitro* results with BaH-1 purified from *B. asper* venom showed that this hemorrhagic toxin was only able to cause detachment of cultured capillary endothelial cells from the substrate, probably due to proteolytic digestion of extracellular matrix components, but without any detectable loss of membrane integrity, as evaluated by the release of intracellular LDH (paper V). These findings indicate that BaH-1 is not directly cytotoxic to endothelial cells, and that their degeneration *in vivo* should be the result of an indirect mechanism, perhaps initiated by the proteolytic degradation of their surrounding basal lamina by the hemorrhagic metalloproteinases. Our conclusions are in contrast with a recent paper reporting that purified hemorrhagic toxins from *Crotalus atrox* and *C. ruber ruber* venoms exert direct cytotoxic action on human endothelial cells in culture (Obrig *et al*., 1993). The conclusion of these authors is based on the decreased neutral red uptake by cells, after 24 hr (for HT-1 and HT-2 of *C. ruber ruber*) or 72 hr (for HT-a and HT-d of *C. atrox*) of exposure to the toxins. However, their cytotoxicity assay utilized two washing steps of the cultures, and thus, it is not clear if the resulting decreased color signal reflects a true cytotoxic effect, as stated by the authors, or a loss of cells due to proteolytic detachment from their substratum. Unfortunately, in their study no attempt was made to describe morphological changes, including detachment, in the cultures treated with the hemorrhagic toxins (Obrig *et al*., 1993). We have shown that detachment is an evident phenomenon in the case of BaH-1 of *B. asper*, implying that the evaluation of direct cytotoxic effects of hemorrhagic toxins should be performed by techniques that avoid washing steps, such as by measuring the release of intracellular markers, or other signs of rapid cell damage, that would be compatible with the *in vivo* observation of capillary disruption within the range of minutes (paper IV).

Our intravital observations of the tissue damage induced by *B. asper* venom confirm and extend the prevalent notion (Ohsaka, 1979; Gutiérrez and Lomonte, 1989; Ownby, 1990) that the toxins responsible for myonecrosis, hemorrhage, and edema, act very rapidly. All these damaging processes were recorded intravitally within only a few minutes after exposure of the tissues to venom (paper IV). This dramatic speed poses a formidable challenge to the search of therapeutical measures that could prevent, or at least, significantly reduce, the tissue damage in human victims of snakebites.

The screening and experimental evaluation of useful neutralizing drugs, as well as the development of improved neutralizing antisera against tissue-damaging toxins, require simple and accurate methods for the quantitative estimation of edema,

hemorrhage, and myonecrosis. While simple methods for edema and hemorrhage quantification are well established, the estimation of muscle damage poses more difficulties. Quantitative histological analysis is ideal, and is currently facilitated by modern computer-assisted microscopic image analyzers. However, even in its simplest forms, quantitative histology is time-consuming, and requires considerable equipment. As an alternative, biochemical methods based on the release of muscle-specific markers, such as creatine kinase (CK), have been utilized.

In this work, we developed a new biochemical assay for the quantification of myonecrosis (paper III), exploiting the known ability of live cells to reduce the tetrazolium compound MTT into a colored formazan product, a reaction commonly utilized in cell culture viability assays (Denizot and Lang, 1986). Other tetrazolium salts, such as nitroblue tetrazolium, have been used to quantify the areas of necrosis in tissue sections of myocardium (Klein *et al*., 1981), and skeletal muscle, in combination with computerized planimetry of the staining pattern (Labbe *et al*., 1988). We found that gastrocnemius muscle homogenates prepared in the presence of 1% Triton X-100, retained the ability to reduce MTT, and that the purple formazan product remained in solution, allowing the direct spectrophotometric measurement of color intensity. The usefulness of this technique in the estimation of myonecrosis was investigated using the venom of *Micrurus nigrocinctus*, which exerts potent myotoxicity in the absence of other tissue-damaging effects such as edema and hemorrhage (Gutiérrez *et al*., 1983). The results of the MTT-reduction assay were compared, by a dose-response analysis, to those obtained by quantitative histological analysis (taken as the reference method), and by two methods based on the quantification of CK activity, in muscle or in plasma, respectively. The residual MTT-reducing activity of muscle rapidly decreased after venom-induced myonecrosis, and the values measured at 24 hr showed a good correlation with the myonecrosis index obtained by quantitative histology. On the other hand, determination of residual CK activity in muscle seemed to overestimate myonecrosis, since with some venom doses, CK activity was almost totally depleted in muscles that still contained a proportion of unaffected fibers, according to the histological reference method.

An interesting observation regarding the use of plasma CK activity was the finding that at high venom doses, where there was virtually a complete myonecrosis (and CK activity depletion) of the injected gastrocnemius, plasma CK levels continued to rise. This implies that at high venom doses, CK release occurs not only at the site of injection, but also at adjacent or distant sites. Thus, plasma CK levels

probably reflect both local and systemic myotoxicity, this property representing an advantage in cases where systemic muscle damage is to be evaluated. However, potential problems of the use of CK as an estimator of muscle damage have been pointed out, such as the possibility of enzyme release in muscle fibers that are not necessarily damaged in an irreversible fashion (Suarez-Kurtz, 1982; Ownby *et al*., 1982).

The validity of the MTT-reducing technique for the quantification of myonecrosis induced by other venoms and myotoxic agents remains to be evaluated, and is currently under study. In particular, it is still not known how other tissue alterations occurring concomitantly with myonecrosis, such as hemorrhage and edema, may affect its performance. At least in the case of *Vipera berus* venom, we observed a fair correlation between muscle damage estimated by plasma CK levels and muscle MTT-reducing activity (paper II).

In addition to *in vivo* techniques for the experimental estimation of myonecrosis, *in vitro* assays utilizing cell cultures could be extremely useful models for the study of myotoxins, their mechanism of action, and neutralization. In particular, cell culture assays would significantly reduce the use of experimental animals in this field of research.

In the course of this investigation, it was observed that *B. asper* myotoxins exhibited cytotoxic activity not only on cultured skeletal muscle myoblasts, but also on several other cell types, including capillary endothelial cells (papers V and VI). Endothelial cells were more susceptible than myoblasts to the cytolytic action of myotoxin II. A simple, non-isotopic cytotoxicity assay, based on the release of lactic dehydrogenase (LDH) from damaged cells was adapted for the *in vitro* study of these myotoxic PLA_2s and their neutralization by different types of agents.

Heparin, previously reported to be able to inhibit the myotoxic action of *B. jararacussu* venom (Melo and Suarez-Kurtz, 1988), was also found to be a potent inhibitor of the cytolytic action of *B. asper* myotoxins *in vitro*, and as well, to neutralize their muscle damaging activity *in vivo* (paper VI). However, a significant limitation in the use of heparin as a neutralizing agent, is its potent anticoagulant action, which may potentiate the hemorrhage caused by *Bothrops* venoms, both due to their potent hemorrhagic toxins and to their ability to affect coagulation (Mandelbaum *et al*., 1984; Selistre *et al*., 1990; Kamiguti *et al*., 1991*a*, 1992; Ouyang *et al*., 1992; Borkow *et al*., 1993). Therefore, we evaluated the neutralizing ability of non-anticoagulant heparin, obtained after affinity chromatography on an immobilized antithrombin support (LA-heparin). It was found that the neutralizing

ability of heparin did not depend on its anticoagulant activity. Thus, LA-heparin represents an interesting possibility for the inhibition of myotoxins in snakebite envenomations.

In screening for the effects of LA-heparin on the myotoxic activity of different venoms, it was found that *B. jararacussu* and *Trimeresurus flavoviridis* venoms were also susceptible to this inhibition, the former confirming the original report by Melo and Suarez-Kurtz (1988). However, no inhibition was observed in the case of other venoms tested, also containing highly basic myotoxins. The three venoms neutralized contain PLA_2 myotoxins that have been well characterized, displaying a high structural homology (Yoshizumi *et al*., 1990; Liu *et al*., 1990; Francis *et al*., 1991; Cintra *et al*., 1993) and immunological cross-reactivity (Lomonte *et al*., 1990*c*).

These results suggested that the observed interaction between heparins and myotoxic PLA_2s could be based not only on a non-specific electrostatic attraction due to the basic character of the enzymes, but that some specific recognition component might additionally be involved. Further structural analysis of the interaction between heparins and myotoxin II (paper VII), showed that the binding does not occur via specific heparin sequences (i.e. as with antithrombin; Lindahl *et al*., 1984), but rather that it depends on cooperative electrostatic interactions involving both N-sulfates and O-sulfates (in 2-O and 6-O positions), the binding efficiency being proportional to the heparin chain length and the overall sulfation. Nevertheless, some specificity for a heparin/heparan sulfate "backbone" in this interaction was suggested by the very weak binding obtained with other highly sulfated GAGs, such as chondroitin sulfate and dermatan sulfate, indicating that a high negative charge is not the only requirement for binding.

The minimal heparin chain lenght capable of interacting with myotoxin II was at the hexasaccharide level, and a good correlation was obtained between the binding and neutralization of cytolytic action. Since molecular weight is important for the pharmacokinetic properties of heparins (Bergonzini *et al*., 1992), these observations suggest that future evaluations of the neutralizing ability of heparin should consider the use of shorter non-anticoagulant fragments (i.e. LMW-LA-heparin, M_r 4-5 Kd) in addition to LA-heparin (M_r 15 Kd).

Studies on the binding and neutralization of *B. asper* myotoxins with heparins provided two interesting basic observations concerning the structure-function relationship of these toxins. First, heparin neutralized the myotoxic action of myotoxin III, without affecting its PLA_2 activity, showing another clear example of

the dissociation between these two actions. Second, the lack of competition between heparin and MAb-3, a neutralizing monoclonal antibody, for the binding to myotoxin II, suggests that both molecules interact with different toxin sites, and therefore, that two different mechanisms for the inhibition of toxic activity exist.

The N-terminal 26 amino acid residues of porcine pancreatic PLA_2 have been shown to interact with heparin (Diccianni *et al*., 1991). We could not detect any interaction between heparin and a 26-mer synthetic peptide of the homologous region of myotoxin II, suggesting that the interaction probably occurs via a different protein site than in pancreatic PLA_2. Other myotoxin protein regions are currently being evaluated in order to identify the possible heparin binding site. We postulated that heparins should bind to a strongly cationic toxin site, likely to be involved in the recognition of negatively-charged cellular target sites (Díaz *et al*., 1991; Rufini *et al*., 1992), and thereby inhibiting the cytolytic action.

Despite the potent inhibitory effect of heparins on the *in vitro* cytolytic action of myotoxic PLA_2s, *in vivo* neutralization of myotoxicity by preincubation tests was less efficient (paper VI). Several possibilities exist to explain this finding, but further work is needed to analyze them. It is likely that high affinity heparin-binding factors compete with myotoxins *in vivo*, reducing its interaction with heparin. The similar neutralizing efficiency of MAb-3 against myotoxin II *in vivo* (myotoxicity) and *in vitro* (cytolytic effect), suggests that the observed difference in the case of heparins may not be due to the cell culture model utilized. Future work will assess the neutralizing efficiency of LA-heparins *in vivo*, by independent administration tests, in comparison to antivenoms, or mixtures of antivenom and heparins. Preliminary observations in the mouse model (unpublished data) indicate that there may be a partial, but significant reduction of muscle damage by early administration of LA-heparin.

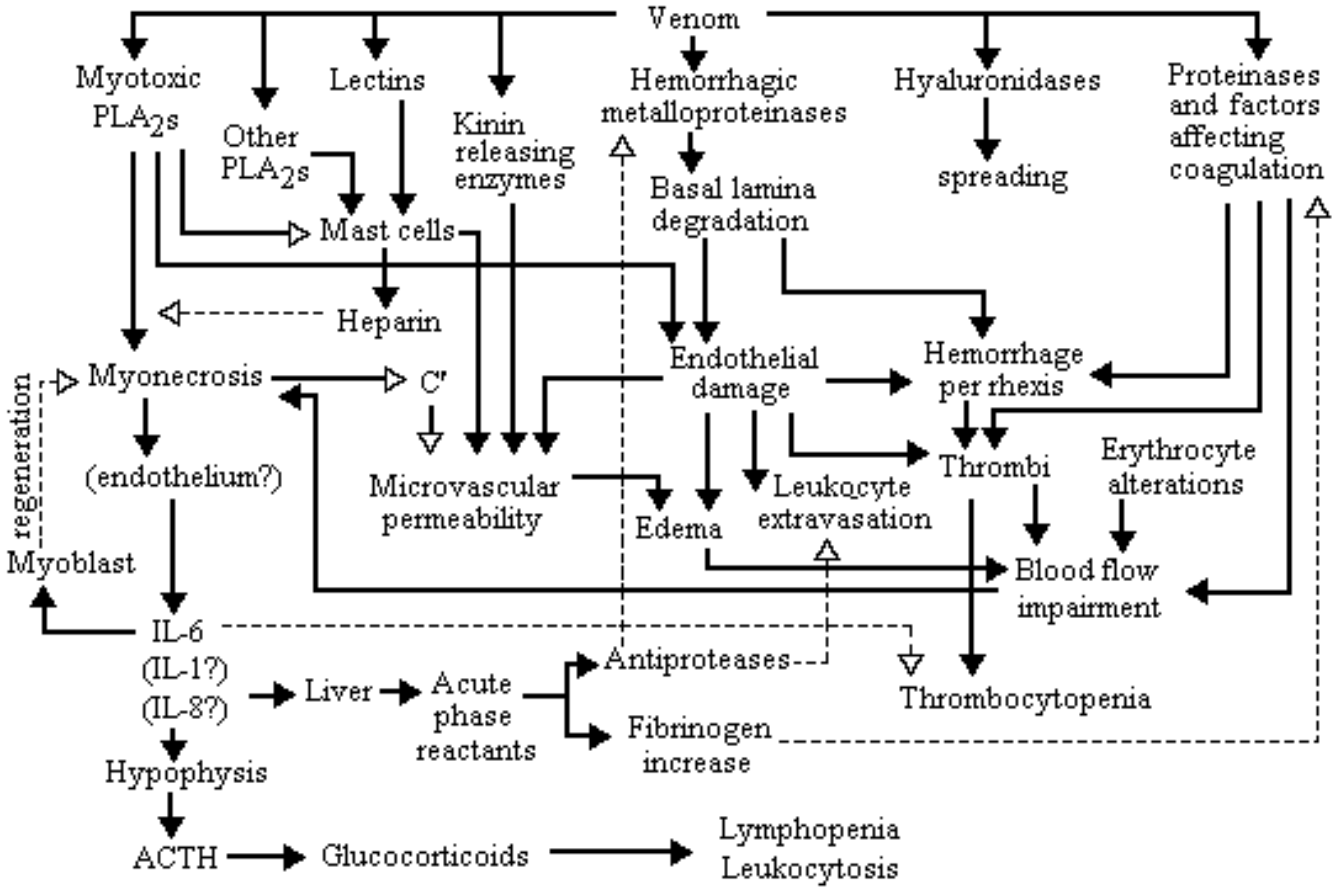

Figure 4: A hypothetical general scheme of some events and interactions in the local tissue damage and inflammatory responses induced by snake venoms. The filled arrowheads denote documented effects, while empty arrowheads denote possible effects. Dashed lines with empty arrowheads indicate inhibitory or counteracting effects.

ACKNOWLEDGEMENTS

My sincere gratitude to my supervisors:

Prof. Lars. Å Hanson, for all his support, interest, encouragement and friendship during this work, admirably transforming a life-threatening snakebite experience into a modest, but fruitful and pleasant, research project.

Dr Andrej Tarkowski, for accepting me in his already numerous student group and nevertheless, guiding me with interest, excellent ideas, and plenty of time through the lab work during these years, with never-failing support and friendship.

Dr Ulf Bagge, for introducing me into the fascinating world of intravital microscopy, and for sharing many hours of exciting experiments at the bench with unforgettable support, friendship, and good humour.

Dr José María Gutiérrez, for guiding the studies performed in Costa Rica, as well as for valuable advice, discussion, support, and friendship in the whole of this work, and a very stimulating and positive collaboration over more than ten years.

My special gratitude also to Lena Svensson, Ing-Marie Nilsson, and Margareta Verdrengh, for their friendship, excellent assistance, teaching, and support, in the lab of the "Rheuma" group. In the same way, to all the personnel of the Instituto Clodomiro Picado, and especially to Marjorie Romero, Gisela Bogarín, Javier Núñez, Guillermo León, Leonel Calderón, and Rodrigo Aymerich, for their important and excellent contribution to the lab work in Costa Rica, as well as to my research colleagues Cecilia Díaz, Alberto Alape, Gustavo Rojas, and Fernando Chaves for many stimulating discussions during this project. The valuable administrative support in both institutes, by Marianne Bäckman, Eva Sjögren, Lotta Malmefeldt, Gertie Flodén, Hilda Herrera, and Rocío Monge, is gratefully appreciated. I would like to thank, as well, all the people at the Department of Clinical Immunology for great hospitality, help, and generosity with another foreign student.

I am very grateful to all the coauthors of the papers included in this work, for a pleasant collaboration and contribution.

I would also like to acknowlege the contributions to different aspects of my work by the following persons: Drs. Monica Thelestam, Ulf Lindahl, Alberto Mantovani, Jeff Esko, Ingemar Björk, Lars O. Andersson, Eva Hurt, Edward Trybala, Ivar Lönnroth, A. Naggi, G. Ronzoni, and W. Murphy.

Thanks to my friends and contemporary PhD students in the "Rheuma" group for a nice working atmosphere, good study (and non-study) sessions, and patience

with a constant favor-asking fellow: Tina Trollmo, Elisabet Josefsson, Yixue Zhao, Nicklas Nilsson, Tomas Bremell, and Arturo Abdelnour. To Arturo, Verónica, and Jerónimo (almost member of our department), my special thanks for unconditional help and friendship.

Thanks to my friends in this and other Departments for making life better for me, Margarita, and Irene, during a sometimes difficult adaptation process in a new cultural and physical environment: Verónica Herías, Pablo Mayorga, Adriana Suárez, Gloria Viboud, Jerónimo Cello, Christina Hessle, Ernesto Moreno, and, very specially, Ingela and Manuel Delgado.

Lastly, but most importantly, thanks to my wife Margarita and my little daughter Irene, for patience, sacrifice, support, and wonderful times during these years; and to my parents-in-law Omar and Gilda, and my mother Rosina, without whom none of this would have been possible.

The financial support from the following entities is gratefully acknowledged: Consejo Nacional de Investigaciones Científicas y Tecnológicas de Costa Rica (CONICIT), Swedish Agency for Research Cooperation with Developing Countries (SAREC), Kabi-Pharmacia, Swedish Medical Research Council (00663), International Foundation for Science (1388/2), Agency for International Development, and University of Costa Rica.

REFERENCES

Abbas, A.K., Lichtman, A.H. and Pober, J.S. (1991) *Cellular and Molecular Immunology*, 417 pp. Philadelphia, W.B. Saunders Company.

Ahuja, M.L. and Singh, G. (1954) Snake bite in India. *Ind. J. Med. Res*. **42**, 661-686.

Ahuja, M.L., Veeraraghavan, N. and Menon, I.G.K. (1946) Action of heparin on the venom of *Echis carinatus*. *Nature* **158**, 878.

Alvarado, J. and Gutiérrez, J.M. (1988) Anticoagulant effect of myotoxic phospholipase A_2 isolated from the venom of the snake *Bothrops asper* (Viperidae). *Rev. Biol. Trop*. **36**, 563-565.

Antunes, E., Rodrigues-Simioni, L. and Prado-Franceschi, J. (1989) Cross-neutralization on the histamine-releasing activity of snake venoms. *Acta Physiol. Pharmacol. Latinoam*. **39**, 431-438.

Araki, S., Ishida, T., Yamamoto, T., Kaji, K. and Hayashi, H. (1993) Induction of apoptosis by hemorrhagic snake venom in vascular endothelial cells. *Biochem. Biophys. Res. Comm.* **190**, 148-153.

Arce, V. (1986) A comparative histochemical study of bulbo-urethral and urethral glands in five rodent species. PhD Thesis. University of Cambridge, U.K.

Atkinson, T.P., White, M.V. and Kaliner, M.A. (1992) Histamine and serotonin. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.), pp.193-209. New York, Raven Press.

Austin, L. and Burgess, A.W. (1991) Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. *J. Neurol. Sci.* **101**, 193-197.

Avila, J.L. and Convit, J. (1976) Physicochemical characteristics of the glycosaminoglycan-lysosomal enzyme interaction *in vitro*. *Biochem. J.* **160**, 129-136.

Bailey, G.S., Al-Joufi, A., Rawat, S. and Smith, D.C. (1991) Neutralization of kinin-releasing enzymes of crotalid venoms by monospecific and polyspecific antivenoms. *Toxicon* **29**, 777-781.

Baker, B., Utaisincharoen, P. and Tu., A.T. (1991) Structure-function relationship of myotoxin *a* using peptide fragments. *Archs. Biochem. Biophys.* **298**, 325-331.

Baker, B., Tu, A.T. and Middlebrook, J.L. (1993) Binding of myotoxin *a* to cultured muscle cells. *Toxicon* **31**, 271-284.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B. and Fox, J.W. (1989) Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. *Archs. Biochem. Biophys.* **275**, 63-71.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B. and Fox, J.W. (1990*a*) Identification of the cleavage sites by a hemorrhagic metalloproteinase in type IV collagen. *Matrix* **10**, 91-97.

Baramova, E.N., Shannon, J.D., Bjarnason, J.B., Gonias, S.L. and Fox, J.W. (1990*b*) Interaction of hemorrhagic metalloproteinases with human α2-macroglobulin. *Biochemistry* **29**, 1069-1074.

Baramova, E.N., Shannon, J.D., Fox, J.W. and Bjarnason, J.B. (1991) Proteolytic digestion of non-collagenous basement membrane proteins by the hemorrhagic metalloproteinase Ht-e from *Crotalus atrox* venom. *Biomed. Biochim. Acta* **50**, 763-768.

Barrantes, A., Solís, V. and Bolaños, R. (1985) Alteración de los mecanismos de la coagulación en el envenenamiento por *Bothrops asper* (terciopelo). *Toxicon* **23**, 399-407.

Benbassat, J. and Shalev, O. (1993) Envenomation by *Echis coloratus* (Mid-East saw-scaled viper): a review of the literature and indications for treatment. *Isr. J. Med. Sci*. **29**, 239-250.

Barnes, J.L. and Abboud, H.E. (1993) Temporal expression of autocrine growth factors corresponds to morphological features of mesangial proliferation in Habu snake venom-induced glomerulonephritis. *Am. J. Pathol*. **143**, 1366-1376.

Bergonzini, G.L., Bianchini, P., Mascellani, G., Osima, B., Parma, B. and Volpi, N. (1992) Different pharmacokinetics, antithrombotic activity and bleeding effects of heparin and two new fragments administered in rat by subcutaneous route. *Arzneim-Forsh/Drug Res.* **42**, 1322-1328.

Bjarnason, J.B. and Fox, J.W. (1988-89) Hemorrhagic toxins from snake venoms. *J. Toxicol.-Toxin Reviews* **7**, 121-209.

Bjarnason, J.B. and Fox, J.W. (1993) Hemorrhagic toxins from snake venoms. Structure, function, target substrates and relationship to mammalian reproductive proteins. Abstracts of the XI European Meeting of the International Society on Toxinology, Arezzo, Italy.

Bjarnason, J.B., Barish, A., Direnzo, G.S., Campbell, R. and Fox, J.W. (1983) Kallikrein-like enzymes from *Crotalus atrox* venom. *J. Biol. Chem.* **258**, 12566-12573.

Bjarnason, J.B., Hamilton, D. and Fox, J.W. (1988) Studies on the mechanism of hemorrhage production by five proteolytic hemorrhagic toxins from *Crotalus atrox* venom. *Biol. Chem. Hoppe-Seyler* **369**, 121-129.

Bober, M.A., Glenn, J.L., Straight, R.C. and Ownby, C.L. (1988) Detection of myotoxin *a*-like proteins in various snake venoms.*Toxicon* **26**, 665-673.

Bolaños, R. and Cerdas, L. (1980) Producción y control de sueros antiofídicos en Costa Rica. *Boln. Of. Sanit. Panam*. **88**, 184-196.

Bonta, I.L., Vargaftig, B.B. and Böhm, G.M. (1979) Snake venoms as an experimental tool to induce and study models of microvessel damage. In: *Handbook of Experimental Pharmacology*, vol.52, *Snake Venoms* (Lee, C.Y., Ed.), pp.629-683. Berlin, Springer-Verlag.

Borkow, G., Gutiérrez, J.M. and Ovadia, M. (1993) Isolation and characterization of synergistic hemorrhagins from the venom of the snake *Bothrops asper*. *Toxicon* **31**, 1137-1150.

Bradley, L.M. (1980) Cell proliferation. In: *Selected Methods in Cellular Immunology* (Mishell, B.B. and Shiigi, S.M., Eds.), p.153-172. San Francisco, Freeman and Co.

Brenes, F., Gutiérrez, J.M. and Lomonte, B. (1987) Immunohistochemical demonstration of the binding of *Bothrops asper* myotoxin to skeletal muscle sarcolemma. *Toxicon* **25**, 574-577.

Brusés, J.L., Capaso, J., Katz, E. and Pilar, G. (1993) Specific *in vitro* biological activity of snake venom myotoxins. *J. Neurochem.* **60**, 1030-1042.

Bultrón, E., Gutiérrez, J.M. and Thelestam, M. (1993*a*) Effects of *Bothrops asper* (terciopelo) myotoxin III, a basic phospholipase A_2, on liposomes and mouse gastrocnemius muscle. *Toxicon* **31**, 217-222.

Bultrón, E., Thelestam, M. and Gutiérrez, J.M. (1993*b*) Effects on cultured mammalian cells of myotoxin III, a phospholipase A_2 isolated from *Bothrops asper* (terciopelo) venom. *Biochim. Biophys. Acta* **1179**, 253-259.

Bussolino, F., De Rossi, M., Sica, A., Colotta, F., Wang, J.M., Bocchietto, E., Padura, I.M., Bosia, A., Dejana, E. and Mantovani, A. (1991) Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. *J. Immunol.* **147**, 2122-2129.

Calhoun, W., Yu, J., Sung, A., Chau, T.T., Marshall, L.A., Weichman, B.M. and Carlson, R.P. (1989) Pharmacologic modulation of D-49 phospholipase A_2-induced paw edema in the mouse. *Agents Actions* **27**, 418-421.

Cameron, D. and Tu, A.T. (1977) Characterization of myotoxin a from the venom of prairie rattlesnake (*Crotalus viridis viridis*). *Biochemistry* **16**, 2546-2553.

Cardoso, J.L.C., Fan, H.W., França, F.O.S., Jorge, M.T., Leite, R.P., Nishioka, S.A., Avila, A., Sano-Martins, I.S., Tomy, S.C., Santoro, M.L., Chudzinski, A.M., Castro, S.C.B., Kamiguti, A.S., Kelen, E.M.A., Hirata, M.H., Mirandola, R.M.S., Theakston, R.D.G. and Warrell, D.A. (1993) Randomized comparative trial of three antivenoms in the treatment of envenoming by lance-headed vipers (*Bothrops jararaca*) in São Paulo, Brazil. *Quart. J. Med.* **86**, 315-325.

Carroll, S.B., Thalley, B.S., Theakston, R.D.G. and Laing, G. (1992) Comparison of the purity and efficacy of affinity purified avian antivenoms with commercial equine crotalid antivenoms. *Toxicon* **30**, 1017-1025.

Castell, J.V., Andus, T., Kunz, D. and Heinrich, P.C. (1989) Interleukin-6: the major regulator of acute-phase protein synthesis in man and rat. *Ann. N.Y. Acad. Sci.* **557**, 87-101.

Castle, N.A., Haylett, D.G. and Jenkinson, D.H. (1989) Toxins in the characterization of potassium channels. *Trends Neurosci.* **12**, 59-65.

Cerami, A. (1992) Inflammatory cytokines. *Clin. Immunol. Immunopathol.* **62**, S3-S10.

Chang, C. and Tseng, K. (1978) Effect of crotamine, a toxin of South American rattlesnake venom, on the sodium channel of murine skeletal muscle. *Br. J. Pharmacol.* **63**, 551-559.

Chaves, F., Gutiérrez, J.M., Lomonte, B. and Cerdas, L. (1989) Histopathological and biochemical alterations induced by intramuscular injection of *Bothrops asper* (terciopelo) venom in mice. *Toxicon* **27**, 1085-1093.

Chen, S.F., Li, S.H., Fei, X. and Wu, Z.L. (1990) Phospholipase A_2-induced lung edema and its mechanism in isolated perfused guinea pig lung. *Inflammation* **14**, 267-273.

Chiu, H.F., Chen, I.J. and Teng, C.M. (1989) Edema formation and degranulation of mast cells by a basic phospholipase A_2 purified from *Trimeresurus mucrosquamatus* snake venom. *Toxicon* **27**, 115-125.

Chwetzoff, S. (1990) On the mode of action of basic phospholipase A_2 from *Naja nigricollis* venom. *Biochim. Biophys. Acta* **1045**, 285-290.

Chwetzoff, S., Tsunasawa, S., Sakiyama, F. and Ménez, A. (1989*a*) Nigexine, a phospholipase A_2 from cobra venom with cytotoxic properties not related to esterase activity. *J. Biol. Chem.* **264**, 13289-13297.

Chwetzoff, S., Couderc, J., Frachon, P. and Ménez, A. (1989*b*) Evidence that the anti-coagulant and lethal properties of a basic phospholipase A_2 from snake venom are unrelated. *FEBS Lett.* **248**, 1-4.

Cintra, A.C.O., Marangoni, S., Oliveira, B. and Giglio, J.R. (1993) Bothropstoxin-I: amino acid sequence and function. *J. Prot. Chem*. **12**, 57-64.

Cirino, G., Peers, S.H., Wallace, J.L. and Flower, R.J. (1989) A study of phospholipase A_2-induced edema in rat paw. *Eur. J. Pharmacol*. **166**, 505-510.

Cirino, G., Cicala, C., Sorrentino, L. and Regoli, D. (1991) Effect of bradykinin antagonists, N^G-monomethyl-L-arginine and L-N^G-nitroarginine on phospholipase A_2 induced oedema in rat paw. *Gen. Pharmacol*. **22**, 801-804.

Coelho, L.K., Silva, E., Espositto, C. and Zanin, M. (1992) Clinical features and treatment of Elapidae bites: report of three cases. *Hum. Exp. Toxicol*. **11**, 135-137.

Cohen, I., Zur, M., Kaminski, E. and de Vries, A. (1970) Isolation and characterization of kinin-releasing enzyme of *Echis coloratus* venom. *Biochem. Pharmacol*. **19**, 785-793.

Collins, M.K.L. and Lopez, A. (1993) The control of apoptosis in mammalian cells. *TIBS* **18**, 307-309.

Condrea, E. and De Vries, A. (1964) Hemolysis and splitting of human erythrocyte phospholipids by snake venoms. *Biochim. Biophys. Acta* **84**, 60-73.

Cramer, E. B. (1992) Cell biology of phagocyte migration from the bone marrow, out of the bloodstream, and across organ epithelia. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.), pp.341-351. New York, Raven Press.

Crosland, R.D. (1989) Development of drug therapies for snake venom intoxication. In: *Natural Toxins: Characterization, Pharmacology, and Therapeutics* (Ownby, C.L. and Odell, G.V., Eds.), pp.165-175. Oxford, Pergamon Press.

Crosland, R.D. (1991) Effect of drugs on the lethality in mice of the venoms and neurotoxins from sundry snakes. *Toxicon* **29**, 613-631.

Cruickshank, A.M., Fraser, W.D., Burns, H.J.G., Van Damme, J. and Shenkin, A. (1990) Response of serum interleukin-6 in patients undergoing elective surgery of varying severity. *Clin. Sci*. **79**, 161-165.

Cull-Candy, S.G., Fohlman, J., Gustavsson, D., Lullmann-Rauch, R. and Thesleff, S. (1976) The effects of taipoxin and notexin on the function and fine structure of the murine neuromuscular junction. *Neuroscience* **1**, 175-180.

d'Albis, A., Couteaux, R., Janmot, C., Roulet, A. and Mira, J.C. (1988) Regeneration after cardiotoxin injury of innervated and denervated slow and fast muscles of mammals: myosin isoform analysis. *Eur. J. Biochem*. **174**, 103-110.

Danishefsky, I., and Steiner, H. (1965) Investigations on the chemistry of heparin. V. Disaccharides obtained after partial hydrolysis. *Biochim. Biophys. Acta* **101**, 37-45.

Davidson, F. and Dennis, E.A. (1990) Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venom to human secreted forms. *J. Mol. Evol.* **31**, 228-238.

Dempfle, C.E., Kohl, R., Harenberg, J., Kirschtein, W., Schlauch, D. and Heene, D.L. (1990) Coagulopathy after snake bite by *Bothrops neuwiedi*: case report and results of *in vitro* experiments. *Blut* **61**, 369-374.

Denizot, F. and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Meth.* **89**, 271-277.

Detrait, J. and Jacob, J. (1988) Antagonists of mouse paw edema induced by habu snake (*Trimeresurus flavoviridis*) venom. *Jap. J. Exp. Med*. **58**, 249-259.

De Vries, A., Rechnic, Y., Moroz, C. and Moav, B. (1963) Prevention of *Echis colorata* venom-induced afibrinogenemia by heparin. *Toxicon* **1**, 241-242.

Dhillon, D. S., Condrea, E., Maraganore, J.M., Heinrikson, R.L., Benjamin, S. and Rosenberg, P. (1987) Comparison of enzymatic and pharmacological activities of lysine-49 and aspartate-49 phospholipases A_2 from *Agkistrodon piscivorus piscivorus* snake venom. *Biochem. Pharmacol*. **36**, 1723-1730.

Díaz, C., Gutiérrez, J.M., Lomonte, B. and Gené, J.A. (1991) The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities. *Biochim. Biophys. Acta* **1070**, 455-460.

Díaz, C., Gutiérrez, J.M. and Lomonte, B. (1992) Isolation and characterization of basic myotoxic phospholipases A_2 from *Bothrops godmani* (Godman's pit viper) snake venom. *Archs. Biochem. Biophys.* **298**, 135-142.

Diccianni, M.B., Mistry, M.J., Hug, K. and Harmony, J.A.K. (1990) Inhibition of phospholipase A_2 by heparin. *Biochim. Biophys. Acta* **1046**: 242-248.

Diccianni, M.B., Lilly, M., McLean, L.R., Balasubramaniam, A. and Harmony, J.A.K. (1991) Heparin prevents the binding of phospholipase A_2 to phospholipid micelles: importance of the amino-terminus. *Biochemistry* **30,** 9090-9097.

Dinarello, C.A. (1992) Role of interleukin-1 and tumor necrosis factor in systemic responses to infection and inflammation. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.), pp.211-232. New York, Raven Press.

Dos Santos, M.C., Gonçalves, L.R.C., Fortes-Dias, C.L., Cury, Y., Gutiérrez, J.M. and Furtado, M.F. (1992) A eficácia do antiveneno botrópico-crotálico na neutralização das principais atividades do veneno de *Bothrops jararacussu*. *Rev. Inst. Med. Trop. São Paulo* **34**, 77-83.

Duchen, L.W., Excell, B.J., Patel, R. and Smith, B. (1974) Changes in motor end-plates resulting from muscle fibre necrosis and regeneration. *J. Neurol. Sci*. **21**, 391-417.

Dufton, M.J. (1993) Kill and cure: the promising future for venom research. *Endeavour* **17**, 138-140.

Dufton, M.J. and Hider, R.C. (1991) The structure and pharmacology of elapid cytotoxins. In: *Snake Toxins* (Harvey, A.L., Ed.), pp.259-302. New York, Pergamon Press.

Emslie-Smith, A. and Harris, J.B. (1989) Lymphopaenia in the inflammatory response caused by nothechis II-5 (Letter). *Toxicon* **27**, 499-500.

Espevik, T. and Nissen-Meyer, J. (1986) A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Meth.* **95**, 99-105.

Estrade, G., Garnier, D., Bernasconi, F. and Donatien, Y. (1989) Embolie pulmonaire et coagulation intravasculaire disséminée aprés une morsure de serpent *Bothrops lanceolatus*. *Arch. Mal. Coeur* **82**, 1903-1905.

Fabiano, R.J. and Tu, A.T. (1981) Purification and biochemical study of viriditoxin, tissue damaging toxin, from prairie rattlesnake venom. *Biochemistry* **20**, 21-27.

Falus, A. and Merétey, K. (1992) Histamine: an early messenger in inflammatory and immune reactions. *Immunol. Today* **13**, 154-156.

Ferrer, X. and Moreno, J.J. (1992) Effects of copper, iron and zinc on oedema formation induced by phospholipase A_2. *Comp. Biochem. Physiol*. **102C**, 325-327.

Ferreira, L.A.F., Henriques, O.B., Lebrun, I., Batista, M.B.C., Prezoto, B.C., Andreoni, A.S.S., Zelnik, R. and Habermehl, G. (1992) Biologically active peptides from *Bothrops jararacussu* venom. In: *Contributions to Autacoid Pharmacology*, pp.209-214. Basel, Birkhäuser Verlag.

Fohlman, J., Eaker, D., Karlsson, E. and Thesleff, S. (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian snake taipan (*Oxyuranus scutellatus*). Isolation, characterization, quaternary structure and pharmacological properties. *Eur. J. Biochem*. **68**, 457-468.

Fraenkel-Conrat, H., Jeng, T.W. and Hsiang, M. (1980) Biological activities of crotoxin and amino acid sequence of crotoxin B. In: *Natural Toxins* (Eaker, D. and Wadström, T., Eds.), pp.561-567. Oxford, Pergamon Press.

Francis, B., Gutiérrez, J.M., Lomonte, B. and Kaiser, I.I. (1991) Myotoxin II from *Bothrops asper* (terciopelo) venom is a lysine-49 phospholipase A_2. *Archs. Biochem. Biophys.* **284**, 352-359.

Franson, R., Patriarca, P. and Elsbach, P. (1974) Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. *J. Lipid Res*. **15**, 380-388.

Furukawa, K. and Ishimaru, S. (1990) Use of thrombin-like snake venom enzymes in the treatment of vascular occlusive diseases. In: *Medical Use of Snake Venom Proteins* (Stocker, K., Ed.), pp.161-174. Boca Raton, CRC Press.

Garfin, S.R., Castilonia, R.R., Mubarak, S.J., Hargens, A.R., Akeson, W.H. and Russell, F.E. (1985) The effect of antivenin on intramuscular pressure elevations induced by rattlesnake venom. *Toxicon* **23**, 677-680.

Gené, J.A., Roy, A., Rojas, G., Gutiérrez, J.M. and Cerdas, L. (1989) Comparative study on coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of Costa Rican crotaline snake venoms and their neutralization by a polyvalent antivenom. *Toxicon* **27**, 841-848.

Gomis-Rüth, F.X., Kress, L.F. and Bode, W. (1993) First structure of a snake venom metalloproteinase: a prototype for matrix metalloproteinases/collagenases. *EMBO J*. **12**, 4151-4157.

Gopalakrishnakone, P., Dempster, D.M., Hagwood, B.J. and Elder, H.Y. (1984) Cellular and mitochondrial changes induced in the structure of murine skeletal muscle by crotoxin, a neurotoxic phospholipase A_2 complex. *Toxicon* **22**, 85-98.

Gould, R.J., Polokoff, M.A., Friedman, P.A., Huang, T.F., Holt, J.C., Cook, J.J. and Niewiarowski, S. (1990) Disintegrins: a family of integrin inhibitory proteins from viper venoms. *Proc. Soc. Exp. Biol. Med*. **195**, 168-171.

Grabar, P. (1986) The historical background of immunology. In: *Basic and Clinical Immunology* (Stites, D., Stobo, J.D. and Wells, J.V., Eds.), p.3. Norwalk, Appleton and Lange.

Greve, H., Cully, Z., Blumberg, P. and Kresse, H. (1988) Influence of chlorate on proteoglycan biosynthesis by cultured human fibroblasts. *J. Biol. Chem.* **263**, 12886-12892.

Gutiérrez, J.M. and Cerdas, L. (1984) Mecanismo de acción de miotoxinas aisladas de venenos de serpientes. *Rev. Biol. Trop*. **32**, 213-222.

Gutiérrez, J.M. and Lomonte, B. (1989) Local tissue damage induced by *Bothrops* snake venoms: a review. *Memorias Inst. Butantan* **51**, 211-223.

Gutiérrez, J.M., Arroyo, O. and Bolaños, R. (1980) Mionecrosis, hemorragia y edema inducidos por el veneno de *Bothrops asper* en ratón blanco. *Toxicon* **18**, 603-610.

Gutiérrez, J.M., Chaves, F., Bolaños, R., Cerdas, L., Rojas, E., Arroyo, O. and Portilla, E. (1981) Neutralización de los efectos locales del veneno de *Bothrops asper* por un antiveneno polivalente. *Toxicon* **19**, 493-500.

Gutiérrez, J.M., Lomonte, B., Portilla, E., Cerdas, L. and Rojas, E. (1983) Local effects induced by coral snake venoms: evidence of myonecrosis after experimental inoculations of venoms from five species. *Toxicon* **21**, 777-783.

Gutiérrez, J.M., Ownby, C.L. and Odell, G.V. (1984*a*) Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon* **22**, 115-128.

Gutiérrez, J.M., Ownby, C.L. and Odell, G.V. (1984*b*) Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Exp. Molec. Pathol.* **40**, 367-379.

Gutiérrez, J.M., Ownby, C.L. and Odell, G.V. (1984*c*) Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops asper* (fer-de-lance). *Toxicon* **22**, 719-731.

Gutiérrez, J.M., Gené, J.A., Rojas, G. and Cerdas, L. (1985) Neutralization of proteolytic and hemorrhagic activities of Costa Rican snake venoms by a polyvalent antivenom. *Toxicon* **23**, 887-893.

Gutiérrez, J.M., Lomonte, B. and Cerdas, L. (1986*a*) Isolation and partial characterization of a myotoxin from the venom of the snake *Bothrops nummifer*. *Toxicon* **24**, 885-894.

Gutiérrez, J.M., Arroyo, O., Chaves, F., Lomonte, B., and Cerdas, L. (1986*b*) Pathogenesis of myonecrosis induced by coral snake (*Micrurus nigrocinctus*) venom in mice. *Br. J. Exp. Pathol*. **67**, 1-12.

Gutiérrez, J.M., Lomonte, B., Chaves, F., Moreno, E. and Cerdas, L. (1986*c*) Pharmacological activities of a toxic phospholipase A isolated from the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* **84C**, 159-164.

Gutiérrez, J.M., Chaves, F., Mata, E. and Cerdas, L. (1986*d*) Skeletal muscle regeneration after myonecrosis induced by *Bothrops asper* (terciopelo) venom. *Toxicon* **24**, 223-231.

Gutiérrez, J.M., Rojas, G., Lomonte, B., Gené, J. A. and Cerdas, L. (1986*e*) Comparative study of the edema-forming activity of Costa Rican snake venoms and its neutralization by a polyvalent antivenom. *Comp. Biochem. Physiol.* **85C**, 171-175.

Gutiérrez, J.M., Chaves, F. and Cerdas, L. (1986*f*) Inflammatory infiltrate in skeletal muscle injected with *Bothrops asper* venom. *Rev. Biol. Trop*. **34**, 209-219.

Gutiérrez, J.M., Rojas, G. and Cerdas, L. (1987) Ability of a polyvalent antivenom to neutralize the venom of *Lachesis muta melanocephala*, a new Costa Rican subspecies of the bushmaster. *Toxicon* **25**, 713-720.

Gutiérrez, J.M., Avila, C., Rojas, E. and Cerdas, L. (1988) An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **26**, 411-413.

Gutiérrez, J.M., Chaves, F., Gené, J.A., Lomonte, B., Camacho, Z. and Schosinsky, K. (1989) Myonecrosis induced by a basic myotoxin isolated from the venom of the snake *Bothrops nummifer* (Jumping viper) from Costa Rica. *Toxicon* **27**, 735-746.

Gutiérrez, J.M., Rojas, G., Lomonte, B., Gené, J.A., Chaves, F., Alvarado, J. and Rojas, E. (1990*a*) Standardization of assays for testing the neutralizing ability of antivenoms (Letter). *Toxicon* **28**, 1127-1129.

Gutiérrez, J.M., Arce, V., Brenes, F. and Chaves, F. (1990*b*) Changes in myofibrillar components after skeletal muscle necrosis induced by a myotoxin isolated from the venom of the snake *Bothrops asper*. *Exp. Mol. Pathol*. **52**, 25-36.

Gutiérrez, J.M., Rojas, G., Pérez, A., Argüello, I. and Lomonte, B. (1991*a*) Neutralization of the venom of coral snake *Micrurus nigrocinctus* by a monovalent antivenom. *Braz. J. Med. Biol. Res*. **24**, 701-710.

Gutiérrez, J.M., Núñez, J., Díaz, C., Cintra, A.C.O., Homsi-Brandeburgo, M.I. and Giglio, J.R. (1991*b*) Skeletal muscle degeneration and regeneration after injection of bothropstoxin-II, a phospholipase A_2 isolated from the venom of the snake *Bothrops jararacussu*. *Exp. Molec. Pathol*. **55**, 217-229.

Gutiérrez, J.M., Rojas, G., Da Silva, N.J. and Núñez, J. (1992) Experimental myonecrosis induced by the venoms of South American *Micrurus* (coral snakes). *Toxicon* **30**, 1299-1302.

Halpert, J. and Eaker, D. (1975) Amino acid sequence of a presynaptic neurotoxin from the venom of *Notechis scutatus scutatus* (Australian tiger snake). *J. Biol. Chem.* **250**, 6990-6997.

Hanson, L.Å. (1991) What is inflammation? Chaos? In: *Clinical Impact of the Monitoring of Allergic Inflammation* (Matsson, L., Ed.), pp.1-5. New York, Academic Press.

Harris, J.B. (1991) Phospholipases in snake venoms and their effects on nerve and muscle. In: *Snake Toxins* (Harvey, A.L., Ed.), pp.91-129. New York, Pergamon Press.

Harris, J.B. and Johnson, M.A. (1978) Further observations on the pathological responses of rat skeletal muscle to toxins isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin. Exp. Pharmacol. Physiol*. **5**, 587-600.

Harris, J.B. and Maltin, C.A. (1982) Myotoxic activity of the crude venom and the principal neurotoxin, taipoxin, of the Australian taipan, *Oxyuranus scutellatus*. *Br. J. Pharmacol*. **76**, 61-75.

Harris, J.B. and Cullen, M.J. (1990) Muscle necrosis caused by snake venoms and toxins. *Electron Microsc. Rev*. **3**, 183-211.

Harris, J.B., Johnson, M.A. and Karlsson, E. (1975) Pathological responses of rat skeletal muscle to a single subcutaneous injection of a toxin isolated from the venom of the Australian tiger snake, *Notechis scutatus scutatus*. *Clin. Exp. Pharmacol. Physiol*. **2**, 383-404.

Harris, J.B., Johnson, M.A. and MacDonell, C.A. (1980) Muscle necrosis induced by some presynaptically active neurotoxins. In: *Natural Toxins* (Eaker, D. and Wadström, T., Eds.), pp.569-578. Oxford, Pergamon Press.

Harris, K., Walker, P.M., Mickle, D.A.G., Harding, R., Gatley, R., Wilson, G.J., Kuzon, B., McKee, N. and Romaschin, A.D. (1986) Metabolic response of skeletal muscle to ischemia. *Am. J. Physiol*. **250**, H213-H220.

Harrison, R.A. and Lachmann, P.J. (1986) Complement technology. In: *Handbook of Experimental Immunology*, vol.1, Immunochemistry (Weir, D.M., Ed.), pp.39.1-39.49.

Harvey, A.L. (1990) Cytolytic toxins. In: *Handbook of Toxinology* (Shier, W.T. and Mebs, D., Eds.), pp.1-66. New York, Marcel Dekker.

Hayes, C.E. and Bieber, A.L. (1986) The effects of myotoxin from midget faded rattlesnake (*Crotalus viridis concolor*) venom on neonatal rat myotubes in cell culture. *Toxicon* **24**, 169-173.

Helmke, S. and Howard, B.D. (1986) Mechanism of inhibition of calcium uptake into sarcoplasmic reticulum by notexin, a neurotoxic and myotoxic polypeptide. *Membrane Biochem*. **6**, 239-253.

Heluany, N.F., Homsi-Brandeburgo, M.I., Giglio, J.R., Prado-Franceschi, J. and Rodrigues-Simioni, L. (1992) Effects induced by bothropstoxin, a component from *Bothrops jararacussu* snake venom, on mouse and chick muscle preparations. *Toxicon* **30**, 1203-1210.

Higginbotham, R.D. (1965) Mast cells and local resistance to Russell's viper venom. *J. Immunol*. **95**, 867-875.

Hirano, T., Akira, S., Taga, T. and Kishimoto, T. (1990) Biological and clinical aspects of interleukin 6. *Immunol. Today* **11**, 443-449.

Hite, L.A., Fox, J.W. and Bjarnason, J.B. (1992*a*) A new family of proteinases is defined by snake venom metalloproteinases. *Biol. Chem. Hoppe-Seyler* **373**, 381-385.

Hite, L.A., Shannon, J.D., Bjarnason, J.B. and Fox, J.W. (1992*b*) Sequence of a cDNA clone encoding the zinc metalloproteinase hemorrhagic toxin *e* from *Crotalus atrox*: evidence for signal, zymogen, and disintegrin-like structures. *Biochemistry* **31**, 6203-6211.

Ho, C.L., Tsai, I.H. and Lee, C.Y. (1986*a*) The role of enzyme activity and charge properties on the presynaptic neurotoxicity and the contracture-inducing activity of snake venom phospholipases A_2. *Toxicon* **24**, 337-345.

Ho, M., Warrell, D.A., Looareesuwan, S., Phillips, R.E., Chanthavanich, P., Karbwang, J., Supanaranond, W., Viravan, C., Hutton, R.A. and Vejcho, S. (1986*b*) Clinical significance of venom antigen levels in patients envenomed by the Malayan pit viper (*Calloselasma rhodostoma*). *Am. J. Trop. Med. Hyg*. **35**, 579-587.

Hoch, R.C., Rodriguez, R., Manning, T., Bishop, M., Mead, P., Shoemaker, W.C. and Abraham, E. (1993). Effects of accidental trauma on cytokine and endotoxin production. *Crit. Care Med*. **21**, 839-845.

Hodges, S.J., Agbaji, A.S., Harvey, A.L. and Hider, R.C. (1987) Cobra cardiotoxins: purification, effects on skeletal muscle and structure/activity relationships. *Eur. J. Biochem*. **165**, 373-383.

Hodgson, D., Gasparini, S., Drevet, P., Ducancel, F., Bouet, F., Boulain, J.C., Harris, J.B. and Ménez, A. (1993) Production of recombinant notechis 11'2L, an enzymatically active mutant of a phospholipase A_2 from *Notechis scutatus scutatus* venom, as directly generated by clevage of a fusion protein produced in *Escherichia coli*. *Eur. J. Biochem*. **212**, 441-446.

Homma, M. and Tu, A.T. (1970) Antivenin for the treatment of local tissue damage due to envenomation by Southeast Asian snakes: ineffectiveness in the prevention of local tissue damage in mice after envenomation. *Am. J. Trop. Med. Hyg*. **19**, 880-884.

Homma, M. and Tu, A.T. (1971) Morphology of local tissue damage in experimental snake envenomation. *Br. J. Exp. Pathol*. **52**, 538-542.

Homsi-Brandeburgo, M.I., Queiroz, L.S., Santo-Neto, H., Rodrigues-Simioni, L. and Giglio, J.R. (1988) Fractionation of *Bothrops jararacussu* snake venom: partial chemical characterization and biological activity of bothropstoxin. *Toxicon* **26**, 615-627.

Hong, S. and Chang, C. (1985) Electrophysiological studies of myotoxin *a*, isolated from prairie rattlesnake (*Crotalus viridis viridis*) venom, on murine skeletal muscles. *Toxicon* **23**, 927-937.

Horigome, K., Hayakawa, M., Inoue, K. and Nojima, S. (1987) Purification and characterization of phospholipase A_2 released from rat platelets. *J. Biochem.* **101**, 625-631.

Huang, H.C. (1984) Release of slow reacting substance from the guinea-pig lung by phospholipases A_2 of *Vipera russelli* snake venom. *Toxicon* **22**, 359-372.

Höök, M., Riesenfeld, J. and Lindahl, U. (1982) N-[^{3}H]Acetyl-labeling, a convenient method for radiolabeling of glycosaminoglycans. *Anal. Biochem.* **119**, 236-245.

Ihrcke, N.S., Wrenshall, L.E., Lindman, B.J. and Platt, J.L. (1993) Role of heparan sulfate in immune system-blood vessel interactions. *Immunol. Today* **14**, 500-505.

Iverius, P.H. (1971) Coupling of glycosaminoglycans to agarose beads (Sepharose 4B). *Biochem. J*. **124**, 677-683..

Jiang, M., Häggblad, J., Heilbronn, E., Rydqvist, B. and Eaker, D. (1987) Some biochemical characteristics and cell membrane actions of a toxic phospholipase A_2 isolated from the venom of the pit viper *Agkistrodon halys* (Pallas). *Toxicon* **25**, 785-792.

Jiménez-Porras, J.M. (1968) Pharmacology of peptides and proteins in snake venoms. *Ann. Rev. Pharmacol*. **8**, 299-318.

Jiménez-Porras, J.M. (1970) Biochemistry of snake venoms. *Clin. Toxicol*. **3**, 389-431.

Johnson, E.K. and Ownby, C.L. (1993) Isolation of a myotoxin from the venom of *Agkistrodon contortrix laticinctus* (broad-banded copperhead) and pathogenesis of myonecrosis induced by it in mice. *Toxicon* **31**, 243-255.

Kaiser, I.I., Gutiérrez, J.M., Plummer, D., Aird, S.D. and Odell, G.V. (1990) The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. *Archs. Biochem. Biophys.* **278**: 319-325.

Kamiguti, A.S., Theakston, R.D.G., Desmond, H. and Hutton, R.A. (1991*a*) Systemic haemorrhage in rats induced by a haemorrhagic fraction from *Bothrops jararaca* venom. *Toxicon* **29**, 1097-1105.

Kamiguti, A.S., Cardoso, J.L., Theakston, R.D., Sano-Martins, I.S., Hutton, R.A., Rugman, F.P., Warrell, D.A. and Hay, C.R. (1991*b*) Coagulopathy and haemorrhage in human victims of *Bothrops jararaca* envenoming in Brazil. *Toxicon* **29**, 961-972.

Kamiguti, A.S., Rugman, F.P., Theakston, R.D.G., Franca, F.O.S., Ishii, H, Hay, C.R.M. and B.I.A.S.G. (1992) The role of venom haemorrhagin in spontaneous bleeding in *Bothrops jararaca* envenoming. *Thromb. Haemost*. **67**, 484-488.

Kerrigan, K.R. (1991) Venomous snakebite in Eastern Ecuador. *Am. J. Trop. Med. Hyg*. **44**, 93-99.

Kihara, H., Uchikawa, R., Hattori, S. and Ohno, M. (1992) Myotoxicity and physiological effects of three *Trimeresurus flavoviridis* phospholipase A_2. *Biochem. Int.* **28**, 895-903.

Kini, R.M. and Iwanaga, S. (1986) Structure-function relationships of phospholipases. II: charge density distribution and the myotoxicity of presynaptically neurotoxic phospholipases. *Toxicon* **24**, 895-905.

Kini, R.M. and Evans, H.J. (1987) Structure-function relationships of phospholipases. The anticoagulant region of phospholipases A_2. *J. Biol. Chem*. **262**, 14402-14407.

Kini, R.M. and Evans, H.J. (1989*a*) A common cytolytic region in myotoxins, hemolysins, cardiotoxins and antibacterial peptides. *Int. J. Peptide Prot. Res*. **34**, 277-286.

Kini, R.M. and Evans, H.J. (1989*b*) A model to explain the pharmacological effects of snake venom phospholipases A_2. *Toxicon* **27**, 613-635.

Kini, R.M. and Evans, H.J. (1992) Sructural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor. *Toxicon* **30**, 265-293.

Kjellén, L. and Lindahl, U. (1991) Proteoglycans: structures and interactions. *Annu. Rev. Biochem*. **60**, 443-475.

Klein, H.H., Puschmann, S., Schaper, J. and Schaper, W. (1981) The mechanism of the tetrazolium reaction in identifying experimental myocardial infarction. *Virchows Arch*. **393**, 287-297.

Kochva, E., Oron, U., Ovadia, M., Simon, T. and Bdolah, A. (1980) Venom glands, venom synthesis, venom secretion and evolution. In: *Natural Toxins* (Eaker, D. and Wadström, T., Eds.), pp.3-12.

Koj, A., Gauldie, J. and Baumann, H. (1993) Biological perspectives of cytokine and hormone networks. In: *Acute Phase Proteins: Molecular Biology, Biochemistry, and Clinical Applications* (Mackiewicz, A., Kushner, I. and Baumann, H., Eds.), pp.275-287. Boca Raton, CRC Press.

Kolb, H. and Kolb-Bachofen, V. (1992) Nitric oxide: a pathogenetic factor in autoimmunity. *Immunol. Today* **13**, 157-160.

Komori, Y. and Sugihara, H. (1988) Biological study of muscle degenerating hemorrhagic factors from the venom of *Vipera aspis aspis* (aspic viper). *Int. J. Biochem*. **20**, 1417-1423.

Kondo, H., Kondo, S., Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom. *Jpn. J. Med. Sci. Biol*. **13**, 43-51.

Kornalík, F. (1991) The influence of snake venom proteins on blood coagulation. In: *Snake Toxins* (Harvey, A.L., Ed.), pp.323-384. New York, Pergamon Press.

Kornalík, F. and Taborska, E. (1989) Cross-reactivity of mono- and polyvalent antivenoms with Viperidae and Crotalidae snake venoms. *Toxicon* **27**, 1135-1142.

Kornalík, F. and Vorlová, Z. (1990) Non-specific therapy of a hemorrhagic diathesis after a bite by a young *Bothrops asper* (barba amarilla): a case report. *Toxicon* **28**, 1497-1501.

Kouyoumdjian, J.A., Harris, J.B. and Johnson, M.A. (1986) Muscle necrosis caused by the sub-units of crotoxin. *Toxicon* **24**, 575-583.

Kozin, F. and Cochrane, C.G. (1992) The contact activation system of plasma. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.), pp.103-121. New York, Raven Press.

Krizaj, I., Bieber, A.L., Ritonja, A. and Gubenšek, F. (1991) The primary structure of ammodytin L, a myotoxic phospholipase A_2 homologue from *Vipera ammodytes* venom. *Eur. J. Biochem.* **202**, 1165-1168.

Labbe, R., Lindsay, T., Gatley, R., Romaschin, A., Mickle, D., Wilson, G., Houle, S. and Walker, P. (1988) Quantitation of postischemic skeletal muscle necrosis: histochemical and radioisotope techniques. *J. Surg. Res.* **44**, 45-53.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Laing, G.D., Theakston, R.D.G., Leite, R.P., da Silva, W.D.D., Warrell, D.A. and B.I.A.S.G. (1992) Comparison of the potency of three Brazilian *Bothrops* antivenoms using *in vivo* rodent and *in vitro* assays. *Toxicon* **30**, 1219-1255.

Lam, B.K. and Austen, K.F. (1992) Leukotrienes: biosynthesis, release, and actions. In: *Inflammation: Basic Principles and Clinical Correlates* (Gallin, J.I., Goldstein, I.M. and Snyderman, R., Eds.), pp.139-147. New York, Raven Press.

Lane, D.A., Denton, J., Flynn, A.M., Thunberg, L., and Lindahl, U. (1984) Anticoagulant activities of heparin oligosaccharides and their neutralization by platelet factor 4. *Biochem. J.* **218**, 725-732.

Larrick, J.W., Yost, J.A. and Kaplan, J. (1978) Snake bite among the Waorani Indians of Eastern Ecuador. *Trans. R. Soc. Trop. Med. Hyg.* **72**, 542-543.

Laure, C. (1975) The primary structure of crotamine. *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 213-215.

Lee, C.Y., Linn, J.S. and Wei, J.W. (1970) Identification of cardiotoxin with cobramine B, direct hemolytic factor and cobra venom cytotoxin (Abstract). *Toxicon* **8**, 140.

Lewis, E.J., Sedgwick, A.D. and Hanahoe, T.H.P. (1992) *In vivo* changes in plasma acute phase protein levels in the rat induced by slow release of IL-1, IL-6 and TNF. *Mediators Inflamm.* **1**, 39-44.

Lidholt, K., Weinke, J.L., Kiser, C.S., Lugemwa, F.N., Bame, K.J., Cheifetz, S., Massagué, J., Lindahl, U. and Esko, J.D. (1992) A single mutation affects both N-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc. Natl. Acad. Sci. USA* **89**, 2267-2271.

Lindahl, U., Cifonelli, A., Lindahl, B. and Rodén L. (1965) The role of serine in the linkage of heparin to protein. *J. Biol. Chem.* **240**, 2817-2820.

Lindahl, U., Thunberg, L., Bäckstrom, G., Riesenfeld, J., Nordling, K. and Björk, I. (1984) Extension and structural variability of the antithrombin-binding sequence in heparin. *J. Biol. Chem*. **259**, 12368-12376.

Lindhardt, R.J., Wang, H.M. and Ampofo, S.A. (1992) New methodologies in heparin structure analysis and the generation of LMW heparins. In: *Heparin and Related Polysaccharides* (Lane, D.A., Björk, I. and Lindahl, U., Eds), pp.37-47. New York, Plenum Press.

Liu, C.S., Chen, J.M., Chang, C.H., Chen, S.W., Teng, C.M. and Tsai, I.H. (1991) The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochim. Biophys. Acta* **1077**, 362-370.

Liu, S.Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S. and Kihara, H. (1990) Purification and amino acid sequence of basic protein II, a Lysine-49 phospholipase A_2 with low activity, from *Trimeresurus flavoviridis* venom. *J. Biochem*. **107**, 400-408.

Lloret, S. and Moreno, J.J. (1993) Oedema formation and degranulation of mast cells by phospholipase A_2 purified from porcine pancreas and snake venoms. *Toxicon* **31**, 949-956.

Lloyd, A.G., Embery, G. and Fowler, L.J. (1971) Studies on heparin degradation-I: preparation of [^{35}S] sulphamate derivatives for studies on heparin degrading enzymes of mammalian origin. *Biochem. Pharmacol*. **20**, 637-648.

Lomonte, B. (1985) Edema-forming activity of bushmaster (*Lachesis muta stenophrys*) and Central American rattlesnake (*Crotalus durissus durissus*) venoms and neutralization by a polyvalent antivenom. *Toxicon* **23**, 173-176.

Lomonte, B. and Gutiérrez, J.M. (1983) La actividad proteolítica de los venenos de serpientes de Costa Rica sobre la caseína. *Rev. Biol. Trop*. **31**, 37-40.

Lomonte, B. and Gutiérrez, J.M. (1989) A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27**, 725-733.

Lomonte, B. and Kahan, L. (1988) Production and partial characterization of monoclonal antibodies to *Bothrops asper* (terciopelo) myotoxin. *Toxicon* **26**, 675-689.

Lomonte, B., Gutiérrez, J.M., Moreno, E. and Cerdas, L. (1987) Antibody neutralization of a myotoxin from the venom of *Bothrops asper* (terciopelo). *Toxicon* **25**, 443-449.

Lomonte, B., Gutiérrez, J.M., Furtado, M.F., Otero, R., Rosso, J.P., Vargas, O., Carmona, E. and Rovira, M.E. (1990*a*) Isolation of basic myotoxins from *Bothrops moojeni* and *Bothrops atrox* snake venoms. *Toxicon* **28**, 1137-1146.

Lomonte, B., Rojas, G., Gutiérrez, J.M. and Ramírez, G. (1990*b*) Isolation of a galactose-binding lectin from the venom of the snake *Bothrops godmani* (Godmann's pit viper). *Toxicon* **28**, 75-81.

Lomonte, B., Furtado, M.F., Rovira, M.E., Carmona, E., Rojas, G., Aymerich, R. and Gutiérrez, J.M. (1990*c*) South American snake venom proteins antigenically-related to *Bothrops asper* myotoxins. *Braz. J. Med. Biol. Res.* **23**, 427-435.

Lomonte, B., Gutiérrez, J.M., Carmona, E. and Rovira, M.E. (1990*d*) Equine antibodies to *Bothrops asper* myotoxin II: isolation from polyvalent antivenom and neutralizing ability. *Toxicon* **28**, 379-384.

Lomonte, B., Gutiérrez, J.M., Rojas, G. and Calderón, L. (1991) Quantitation by enzyme-immunoassay of antibodies against *Bothrops* myotoxins in four commercially-available antivenoms. *Toxicon* **29**, 695-702.

Lomonte, B., Gutiérrez, J.M., Ramírez, M. and Díaz, C. (1992) Neutralization of myotoxic phospholipases A_2 from the venom of the snake *Bothrops asper* by monoclonal antibodies. *Toxicon* **30**, 239-245.

Lwin, M., Warrell, D.A., Phillips, R.E., Swe, T.N., Pe, T. and Lay, M.M. (1985) Bites by Russell's viper (*Vipera russelli siamensis*) in Burma: haemostatic, vascular, and renal disturbances and response to treatment. *Lancet* **ii**, 1259-1263.

Lwin, M., Swe, T.N., Mu, M.A., Than, T., Than, T. and Pe, T. (1989) Heparin therapy in Russell's viper bite victims with impending DIC (a controlled trial). *Southeast Asian J. Trop. Med. Publ. Hlth*. **20**, 271-277.

Lycke, E., Johansson, M., Svennerholm, B. and Lindahl, U. (1991) Binding of herpex simplex virus to cellular heparan sulphate, an initial step in the adsorption process. *J. Gen. Virol.* **72**, 1131-1137.

Mackiewicz, A., Ganapathi, M.K., Schultz, D., Samols, D., Reese, J. and Kushner, I. (1988) Regulation of rabbit acute phase protein biosynthesis by monokines. *Biochem. J*. **253**, 851-857.

Maccarana, M., Casu, B. and Lindahl, U. (1993) Minimal sequence in heparin/heparan sulfate required for binding of basic fibroblast growth factor (FGF-2). *J. Biol. Chem*. **268**, 23898-23905.

Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemical quantitation of antigens by radial immunodiffusion. *Immunochemistry* **2**, 235-254.

Mandelbaum, F.R., Assakura, M.T. and Reichl, A.P. (1984) Characterization of two hemorrhagic factors isolated from the venom of *Bothrops neuwiedi* (jararaca pintada) *Toxicon* **22**, 193- 206.

Maraganore, J.M., Merutka, G., Cho, W., Welches, W., Kézdy, F.J. and Heinrikson, R.L. (1984) A new class of phospholipases A_2 with lysine in place of aspartate 49. *J. Biol. Chem*. **259**, 13839-13843.

Marshall, L.A., Chang, J.Y., Calhoun, W., Yu, J. and Carlson, R.P. (1989) Preliminary studies on phospholipase A_2-induced mouse paw edema as a model to evaluate antiinflammatory agents. *J. Cell. Biochem*. **40**, 147-155.

Martz, W. (1992) Plants with a reputation against snakebite. *Toxicon* **30**, 1131-1142.

Maruyama, M., Sugiki, M., Yoshida, E., Shimaya, K. and Mihara, H. (1992) Broad substrate specificity of snake venom fribrinolytic enzymes: possible role in haemorrhage. *Toxicon* **30**, 1387-1397.

McLoon, L.K., Bauer, G. and Wirtschafter, J. (1991) Quantification of muscle loss in the doxorubicin-treated orbicularis oculi of the monkey. Effect of local injection of doxorubicin into the eyelid. *Invest. Ophtalmol. Visual Sci*. **32**, 1667-1673.

Mebs, D. (1970) A comparative study of enzyme activities in snake venoms. *Int. J. Biochem*. **1**, 335-342.

Mebs, D. (1986) Myotoxic activity of phospholipases A_2 isolated from cobra venoms: neutralization by polyvalent antivenoms. *Toxicon* **24**, 1001-1008.

Mebs, D. (1990) Venom components with other important biological activities. In: *Handbook of Toxinology* (Shier, W.T. and Mebs, D., Eds.), pp.761-777. New York, Marcel Dekker.

Mebs, D. and Samejima, Y. (1986) Isolation and characterization of myotoxic phospholipases A_2 from crotalid venoms. *Toxicon* **24**, 161-168.

Mebs, D. and Ownby, C.L. (1990) Myotoxic components of snake venoms: their biochemical and biological activities. *Pharmac. Ther*. **48**, 223-236.

Mebs, D., Ehrenfeld, M. and Samejima, Y. (1983) Local necrotizing effect of snake venoms on skin and muscle: relationship to serum creatine kinase. *Toxicon* **21**, 393-404.

Mebs, D., Pohlmann, S. and Von Tenspolde, W. (1988) Snake venom hemorrhagins: neutralization by commercial antivenoms. *Toxicon* **26**, 453-458.

Mehrtens, J. (1987) *Living snakes of the world*, 480 pp. New York, Sterling Publishing.

Melo, P.A. and Suarez-Kurtz, G. (1987) Interaction of *Bothrops* venoms and antivenin on the release of creatine kinase from skeletal muscle. *Braz. J. Med. Biol. Res*. **20**, 821-824.

Melo, P.A. and Suarez-Kurtz, G. (1988) Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: antagonism by heparin and by the serum of South American marsupials. *Toxicon* **26**, 87-95.

Melo, P.A., Homsi-Brandeburgo, M.I., Giglio, J.R. and Suarez-Kurtz, G. (1993) Antagonism of the myotoxic effect of *Bothrops jararacussu* venom and bothropstoxin by polyanions. *Toxicon* **31**, 285-291.

Ménez, A. (1991) Immunology of snake toxins. In: *Snake Toxins* (Harvey, A.L., Ed.), pp.35-90. New York, Pergamon Press.

Mengden, G. (1983) The taxonomy of Australian elapid snakes. *Records Australian Museum* **35**, 195-222.

Menger, M.D. and Lehr, H.A. (1993) Scope and perspectives of intravital microscopy: bridge over from *in vitro* to *in vivo*. *Immunol. Today* **14**, 519-522.

Moreira, L., Borkow, G., Ovadia, M. and Gutiérrez, J.M. (1994) Pathological changes induced by BaH1, a hemorrhagic proteinase isolated from *Bothrops asper* (terciopelo) snake venom, on mouse capillary blood vessels. *Toxicon* (in press).

Moreno, J.J., Ferrer, X., Ortega, E. and Carganico, G. (1992) PLA_2-induced oedema in rat skin and histamine release in rat mast cells. Evidence for involvement of lysophospholipids in the mechanism of action. *Agents Actions* **36**, 258-263.

Morgan, D.W., Anderson, C.M. and Meyers, K.P. (1993) Recombinant human type II phospholipase A_2 lacks edema producing activity in rat. *Eur. J. Pharmacol*. **235**, 45-50.

Mors, W.B., Nascimento, M.C., Parente, J.P., Da Silva, M.H., Melo, P.A. and Suarez-Kurtz, G. (1989) Neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of the plant *Eclipta prostrata* (Asteraceae). *Toxicon* **27**, 1003-1009.

Moura-da-Silva, A.M., Desmond, H., Laing, G. and Theakston, R.D.G. (1991) Isolation and comparison of myotoxins isolated from venoms of different species of *Bothrops* snakes. *Toxicon* **29**, 713-723.

Moura-da-Silva, A.M., Paine, M.J.I., Theakston, R.D.G. and Crampton, J.M. (1993) Cloning and sequence analysis of Kenyan *Echis carinatus leakeyi* metalloprotease genes. Abstracts of the XI European Meeting of the International Society on Toxinology, Arezzo, Italy.

Nagasawa, K., Inoue, Y., and Kamata, T. (1977) Solvolytic desulfation of glycosaminoglycuronan sulfates with dimetyl sulfoxide containing water or methanol. *Carb. Res.* **58**, 47-55.

Nair, X., Nettleton, D., Clever, D., Tramposch, K.M., Ghosh, S. and Franson, R.C. (1993) Swine as a model of skin inflammation: phospholipase A_2-induced inflammation. *Inflammation* **17**, 205-215.

Nakada, F., Uezu, N., Ohshiro, M. and Miyagi, K. (1980) Relation between serum creatine phosphokinase activity and the amount of Habu (*Trimeresusrus flavoviridis*) snake venom injected in thigh muscle in rabbits. *Toxicon* **18**, 351-355.

Nakada, K., Nakada, F., Ito, E. and Inoue, F. (1984) Quantification of myonecrosis and comparison of necrotic activity of snake venoms by determination of creatine phosphokinase activity in mice sera. *Toxicon* **22**, 921-930.

Nakagawa, M., Nakanishi, K., Darko, L.L. and Vick, J.A. (1982) Structures of cabenegrins A-I and A-II, potent anti-snake venoms. *Tetrahedron Lett*. **23**, 3855-3858.

Nelle, M., Boeije, L. and Aarden, L. (1988) Functional discrimination between interleukin-6 and interleukin-1. *Eur. J. Immunol.* **18**, 1535-1540.

Neves, P.C.A., Neves, M.C.A., Cruz, A.B., Sant'Ana, A.E.G., Yunes, R.A. and Calixto, J.B. (1993) Differential effects of *Mandevilla velutina* compounds on paw edema induced by phospholipase A_2 and phospholipase C. *Eur. J. Pharmacol*. **243**, 213-219.

Ng, R.H. and Howard, B.D. (1980) Mitochondria and sarcoplasmic reticulum as model targets for neurotoxic and myotoxic phospholipases A_2. *Proc. Natl. Acad. Sci. USA* **77**, 1346-1350.

Nishioka, S.A. and Silveira, P.V.P. (1992) A clinical and epidemiologic study of 292 cases of lance-headed viper bite in a Brazilian teaching hospital. *Am. J. Trop. Med. Hyg*. **47**, 805-810.

Obrig, T.G., Louise, C.B., Moran, T.P., Mori, N. and Tu, A.T. (1993) Direct cytotoxic effects of hemorrhagic toxins from *Crotalus ruber ruber* and *Crotalus atrox* on human vascular endothelial cells, *in vitro*. *Microvasc. Res*. **46**, 412-416.

Oguchi, Y., Suda, T., Suzuki, T., Ohtani, Y. and Takahashi, H. (1986) Isolation and physiological action of capillary permeability increasing-enzyme from the venom of *Agkistrodon caliginosus*. *Adv. Exp. Med. Biol*. **198**, 557-564.

Ohsaka, A. (1979) Hemorrhagic, necrotizing and edema-forming effects of snake venoms. In: *Handbook of Experimental Pharmacology*, vol.52, *Snake Venoms* (Lee, C.Y., Ed.), pp.480-546. Berlin, Springer-Verlag.

Ohsaka, A., Just, M. and Habermann, E. (1973) Action of snake venom hemorrhagic principles on isolated glomerular basement membrane. *Biochim. Biophys. Acta* **323**, 415-428.

Olwin, B.B. and Rapraeger, A. (1992) Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. *J. Cell. Biol.* **118**, 631-639.

Oshio, C. (1984) The behaviour of platelets in capillary hemorrhage induced by snake venom. *Keio J. Med*. **33**, 83-97.

Ohtani, Y. and Takahashi, H. (1983) Purification of a capillary permeability increasing-enzyme from the venom of *Agkistrodon caliginosus* (kankoku-mamushi). *Toxicon* **21**, 871-878.

Ouchterlony, Ö. and Nilsson, L.Å. (1978) Immunodiffusion and immunoelectrophoresis. In: *Handbook of Experimental Immunology* (ed. D.M. Weir), pp.19.1-19.44. Oxford, Blackwell Scientific Publications.

Ouyang, C., Teng, C.M. and Huang, T.F. (1992) Characterization of snake venom components acting on blood coagulation and platelet function. *Toxicon* **30**, 945-966.

Ownby, C.L. (1990) Locally acting agents: myotoxins, hemorrhagic toxins and dermonecrotic factors. In: *Handbook of Toxinology* (Shier, W.T. and Mebs, D., Eds.), pp.602-654. New York, Marcel Dekker.

Ownby, C.L. and Colberg, T. (1986) Ability of polyvalent (Crotalidae) antivenom to neutralize local myonecrosis induced by *Crotalus atrox* venom. *Toxicon* **24**, 201-203.

Ownby, C.L. and Colberg, T.R. (1988) Classification of myonecrosis induced by snake venoms: venoms from the prairie rattlesnake (*Crotalus viridis viridis*), Western diamondback rattlesnake (*Crotalus atrox*) and the Indian cobra (*Naja naja naja*). *Toxicon* **26**, 459-474.

Ownby, C.L. and Geren, C.R. (1987) Pathogenesis of hemorrhage induced by hemorrhagic proteinase IV from timber rattlesnake (*Crotalus horridus horridus*) venom. *Toxicon* **25**, 517-526.

Ownby, C.L. and Li, Q. (1993) Molecular cloning of the gene encoding a myotoxin from the venom of the broad-banded copperhead (*Agkistrodon contortrix laticinctus*). Abstracts of the XI European Meeting of the International Society on Toxinology, Arezzo, Italy.

Ownby, C.L., Cameron, D. and Tu, A.T. (1976) Isolation of myotoxic component from rattlesnake (*Crotalus viridis viridis*) venom. Electron microscopic analysis of muscle damage. *Am. J. Pathol*. **85**, 149-166.

Ownby, C.L., Bjarnason, J.W. and Tu, A.T. (1978) Hemorrhagic toxins from rattlesnake (*Crotalus atrox*) venom. Pathogenesis of hemorrhage induced by three purified toxins. *Am. J. Pathol*. **93**, 201-218.

Ownby, C.L., Woods, W.M. and Odell, G.V. (1979) Antiserum to myotoxin from praire rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon* **17**, 373-380.

Ownby, C.L., Gutiérrez, J.M., Colberg, T.R. and Odell, G.V. (1982) Quantitation of myonecrosis induced by myotoxin *a* from prairie rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon* **20**, 877-885.

Ownby, C.L., Odell, G.V., Woods, W.M. and Colberg, T.R. (1983*a*) Ability of antiserum to myotoxin *a* from prairie rattlesnake (*Crotalus viridis viridis*) venom to neutralize local myotoxicity and lethal effects of myotoxin *a* and homologous crude venom. *Toxicon* **21**, 35-45.

Ownby, C.L., Odell, G.V. and Theakston, R.D.G. (1983*b*) Detection of antibodies to myotoxin *a* and prairie rattlesnake (*Crotalus viridis viridis*) venom in three antisera using enzyme-linked immunosorbent assay and immunodiffusion. *Toxicon* **21**, 849-855.

Ownby, C.L., Colberg, T. and Odell, G.V. (1984*a*) A new method for quantitating hemorrhage induced by rattlesnake venoms: ability of polyvalent antivenom to neutralize hemorrhagic activity. *Toxicon* **22**, 227-233.

Ownby, C.L., Colberg, T.R., Claypool, P.L. and Odell, G.V. (1984*b*) *In vivo* test of the ability of antiserum to myotoxin *a* from prairie rattlesnake (*Crotalus viridis viridis*) venom to neutralize local myonecrosis induced by myotoxin *a* and homologous crude venom. *Toxicon* **22**, 99-105.

Ownby, C.L., Colberg, T.R. and Odell, G.V. (1985) Ability of a mixture of antimyotoxin *a* serum and polyvalent (Crotalidae) antivenin to neutralize myonecrosis, hemorrhage and lethality induced by prairie rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon* **23**, 317-324.

Ownby, C.L., Colberg, T.R. and Odell, G.V. (1986) *In vivo* ability of antimyotoxin *a* serum plus polyvalent (Crotalidae) antivenom to neutralize prairie rattlesnake (*Crotalus viridis viridis*) venom. *Toxicon* **24**, 197-200.

Ownby, C.L., Nika, T., Imai, K. and Sugihara, H. (1990) Pathogenesis of hemorrhage induced by bilitoxin, a hemorrhagic toxin isolated from the venom of the common cantil (*Agkistrodon bilineatus bilineatus*). *Toxicon* **28**, 837-846.

Ownby, C.L., Fletcher, J.E. and Colberg, T. (1993) Cardiotoxin 1 from cobra (*Naja naja atra*) venom causes necrosis of skeletal muscle *in vivo*. *Toxicon* **31**, 697-709.

Paine, M.J., Desmond, H.P., Theakston, R.D. and Crampton, J.M. (1992) Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from *Bothrops jararaca* venom. *J. Biol. Chem*. **267**, 22869-22876.

Pedersen, J.Z., Cesaroni, P. and Rufini, S. (1993) Phospholipase-like myotoxins from snake venoms. Abstracts of the XI European Meeting of the International Society on Toxinology, Arezzo, Italy.

Pejler, G., Lindahl, U., Larm, O., Scholander, E., Sandgren, E., and Lundblad, A. (1988) Monoclonal antibodies specific for oligosaccharides prepared by partial nitrous acid deamination of heparin. *J. Biol. Chem.* **263**, 5197-5201.

Perkins, J.R., Parker, C.E. and Tomer, K.B. (1993) The characterization of snake venoms using capillary electrophoresis in conjunction with electrospray mass spectrometry: black mambas. *Electrophoresis* **14**, 458-468.

Pluskal, M.G., Harris, J.B., Pennington, R.J. and Eaker, D. (1978) Some biochemical responses of rat skeletal muscle to a single subcutaneous injection of a toxin (notexin) isolated from the venom of the Australian tiger snake *Notechis scutatus scutatus*. *Clin. Exp. Pharmacol. Physiol*. **5**, 131-141.

Ponton, J., Regulez, P. and Cisterna, R. (1983) Comparison of three tests for measuring footpad swelling in the mouse. *J. Immunol. Meth.* **63**, 139-143.

Preston, S., Davis, C.E. and Harris, J.B. (1990) The assessment of muscle fibre loss after the injection of the venom of *Notechis scutatus* (Australian tiger snake). *Toxicon* **28**, 201-214.

Pruzanski, W. and Vadas, P. (1991) Phospholipase A_2: a mediator between proximal and distal effectors of inflammation. *Immunol. Today* **12**, 143-146.

Pugh, R.N.H. and Theakston, R.D.G. (1980) Incidence and mortality of snake bite in savanna Nigeria. *Lancet* **ii**, 1181-1183.

Queiroz, L.S., Santo Neto, H., Rodrigues-Simioni, L. and Prado-Franceschi, J. (1984) Muscle necrosis and regeneration after envenomation by *Bothrops jararacussu* snake venom. *Toxicon* **22**, 339-346.

Queiroz, L.S., Santo Neto, H., Assakura, M., Reichl, A.P. and Mandelbaum, F.R. (1985*a*) Pathological changes in muscle caused by haemorrhagic and proteolytic factors from *Bothrops jararaca* snake venom. *Toxicon* **23**, 341-345.

Queiroz, L.S., Santo-Neto, H., Assakura, M.T., Reichl, A.P. and Mandelbaum, F.R. (1985*b*) Muscular lesions induced by a hemorrhagic factor from *Bothrops neuwiedi* snake venom. *Braz. J. Med. Biol. Res*. **18**, 337-340.

Raphael, S.S. (1983) *Lynch's Medical Laboratory Technology*, 845 pp. Philadelphia, W.B. Saunders Company.

Rees, B. and Bilwes, A. (1993) Three-dimensional structures of neurotoxins and cardiotoxins. *Chem. Res. Toxicol*. **6**, 385-406.

Reid, H.A. and Theakston, R.D.G. (1983) The management of snake bite. *Bull. W.H.O*. **61**, 885-895.

Reid, H.A., Thean, P.C. and Martin, W.J. (1963) Specific antivenene and prednisone in viper-bite poisoning: controlled trial. *Br. Med. J*. **II**, 1378-1380.

Reisfeld, R.A., Lewis, U.J. and Williams, D.E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195**, 281-283.

Rezende, N.A., Amaral, C.F., Bambirra, E.A., Lachatt, J.J. and Coimbra, T.M. (1989) Functional and histopathological renal changes induced in rats by *Bothrops jararaca* venom. *Braz. J. Med. Biol. Res*. **22**, 407-416.

Robeva, A., Politi, V., Shannon, J.D., Bjarnason, J.B. and Fox, J.W. (1991) Synthetic and endogenous inhibitors of snake venom metalloproteinases. *Biomed. Biochim. Acta* **50**, 769-773.

Rocha e Silva, M., Beraldo, W.T. and Rosenfeld, G. (1949) Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulins by snake venoms and trypsin. *Amer. J. Physiol*. **156**, 261-273.

Rodén, L., Ananth, S., Campbell, P., Curenton, T., Ekborg, G., Manzella, S., Pillion, D. and Meezan, E. (1992) In: *Heparin and Related Polysaccharides* (Lane, D.A., Björk, I. and Lindahl, U., Eds), pp.1-20. New York, Plenum Press.

Roitt, I.M., Brostoff, J. and Male, D. K. (1993) *Immunology*. St. Louis, Mosby.

Rojas, G., Gutiérrez, J.M., Gené, J.A., Gómez, M. and Cerdas, L. (1987) Neutralización de las actividades tóxicas y enzimáticas de cuatro venenos de serpientes de Guatemala y Honduras por el antiveneno polivalente producido en Costa Rica. *Rev. Biol. Trop*. **35**, 59-67.

Rosenberg, P. (1986) The relationship between enzymatic activity and pharmacological properties of phospholipases in natural poisons. In: *Natural Toxins* (Harris, J.B., Ed.), pp.129-174. Oxford, Clarendon Press.

Rosenberg, P. (1990) Phospholipases. In: *Handbook of Toxinology* (Shier, W.T. and Mebs, D., Eds.), pp.67-277. New York, Marcel Dekker.

Rosenfeld, G. (1971) Symptomatology, pathology and treatment of snake bites in South America. In: *Venomous Animals and their Venoms* (Bücherl, W., Buckley, E.E. and Deulofeu, V., Eds.), vol.2, pp-345-384. New York, Academic Press.

Rot, A. (1992) Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol. Today* **13**, 291-294.

Rothschild, A.M. and Rothschild, Z. (1979) Liberation of pharmacologically active substances by snake venoms. In: *Handbook of Experimental Pharmacology*, vol.52, *Snake Venoms* (Lee, C.Y., Ed.), pp.591-628. Berlin, Springer-Verlag.

Rovira, M.E., Carmona, E. and Lomonte, B. (1992) Immunoenzymatic quantitation of antibodies to *Bothrops asper* myotoxins after polyvalent antivenom administration in mice. *Braz. J. Med. Biol. Res.* **25**, 23-33.

Rowan, E.G., Harvey, A.L. and Ménez, A. (1991) Neuromuscular effects of nigexine, a basic phospholipase A_2 from *Naja nigricollis* venom. *Toxicon* **29**, 371-374.

Rufini, S., Cesaroni, P., Desideri, A., Farias, R., Gubensek, F., Gutiérrez, J.M., Luly, P., Massoud, R., Morero, R. and Pedersen, J.Z. (1992) Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. *Biochemistry* **31**, 12424-12430.

Russell, F.E., Ruzic, N. and Gonzalez, H. (1973) Effectiveness of antivenin (Crotalidae) polyvalent following injection of *Crotalus* venom. *Toxicon* **11**, 461-464.

Russell, F.E., Carlson, R.W., Wainschel, J. and Osborne, A.H. (1975) Snake venom poisoning in the United States. Experiences with 550 cases. *J.A.M.A.* **233**, 341-344.

Sáez-Llorens, X. and Lagrutta, F. (1993) The acute phase host reaction during bacterial infection and its clinical impact in children. *Pediatr. Infect. Dis. J.* **12**, 83-87.

Samel, M., Siigur, E. and Siigur, J. (1987) Purification and characterization of two arginine ester hydrolases from *Vipera berus berus* (common viper) venom. *Toxicon* **25**, 379-388.

Scarborough, R.M., Rose, J.W., Naughton, M.A., Phillips, D.R., Nannizzi, L., Arfsten, A., Campbell, A.M. and Charo, I.F. (1993) Characterization of the integrin specificities of disintegrins isolated from American pit viper venoms. *J. Biol. Chem.* **268**, 1058-1065.

Schaeffer, R.C., Briston, C., Chilton, S.M. and Carlson, R.W. (1986) Disseminated intravascular coagulation following *Echis carinatus* venom in dogs: effects of a synthetic thrombin inhibitor. *J. Lab. Clin. Med.* **107**, 488-497.

Scott, D.L., Achari, A., Vidal, J.C. and Sigler, P.B. (1992) Crystallographic and biochemical studies of the (inactive) Lys-49 phospholipase A_2 from the venom of *Agkistrodon piscivorus piscivorus*. *J. Biol. Chem.* **267**, 22645-22657.

Seckinger, P., Lowenthal, J.W., Williamson, K., Dayer, J.M. (1987) A urine inhibitor of interleukin-1 activity that blocks ligand binding. *J. Immunol.* **139**, 1546-1549.

Selistre, H.S., Queiroz, L.S., Cunha, O.A.B., De Souza, G.E.P. and Giglio, J.R. (1990) Isolation and characterization of hemorrhagic, myonecrotic and edema-inducing toxins from *Bothrops insularis* (jararaca ilhoa) snake venom. *Toxicon* **28**, 261-273.

Sen, S. (1992) Programmed cell death: concept, mechanism and control. *Biol. Rev.* **67**, 287-319.

Shah, P.K.D., Chittora, M.D., Shekhawat, J.S., Khangarrot and Vyas, M.M. (1986) Role of heparin in the management of snake (*Echis carinatus*) bite cases. *J. Assoc. Phys. India* **34**, 621-623.

Shannon, J.D., Baramova, E.N., Bjarnason, J.B. and Fox, J.W. (1989) Amino acid sequence of a *Crotalus atrox* venom metalloproteinase which cleaves type IV collagen and gelatin. *J. Biol. Chem.* 264, 11575-11583.

Sharp, N.J.H., Kornegay, J.N., Bartlett, R.J., Hung, W.Y. and Dykstra, M.J. (1993) Notexin-induced muscle injury in the dog. *J. Neurol. Sci.* **116**, 73-81.

Shively, J.E. and Conrad, H.E. (1976) Formation of anhydrosugars in the chemical depolymerization of heparin. *Biochemistry* **15**, 3932-3942.

Smith, D.C., Reddi, K.R., Laing, G., Theakston, R.G.D. and Landon, J. (1992) An affinity purified ovine antivenom for the treatment of *Vipera berus* envenoming. *Toxicon* **30**, 865-871.

Stocker, K. (1990*a*) Snake venom proteins affecting hemostasis and fibrinolysis. In: *Medical Use of Snake Venom Proteins* (Stocker, K., Ed.), pp.97-160. Boca Raton, CRC Press.

Stocker, K. (1990*b*) Application of snake venom proteins in the diagnosis of hemostatic disorders. In: *Medical Use of Snake Venom Proteins* (Stocker, K., Ed.), pp.213-252. Boca Raton, CRC Press.

Suarez-Kurtz, G. (1982) Release of creatine kinase from frog muscle by osmotic changes. *Am. J. Physiol*. **242**, C398-C403.

Swaroop, S. and Grab, B. (1954) Snakebite mortality in the world. *Bull. W.H.O.* **10**, 35-76.

Swe, T.N., Lwin, M., Han, K.E. and Pe, T. (1992) Heparin therapy in Russell's viper bite victims with disseminated intravascular coagulation: a controlled trial. *Southeast Asian J. Trop. Med. Publ. Hlth*. **23**, 282-287.

Syu, W.J. and Kahan, L. (1987) Use of protein-stained immunoblots for unequivocal identification of antibody specificities. *J. Immunol. Meth.* **103**, 247-252.

Takacs, Z., Retzios, A.D. and Markland, F.S. (1992) A hemorrhagic (basement membrane degrading) zinc metalloproteinase from Southern copperhead venom. *Matrix* (Suppl. No.1), 101-103.

Takeya, H., Oda, K., Miyata, T., Omori-Satoh, T. (1990) The complete amino acid sequence of the high molecular mass hemorrhagic protein HR1B isolated from the venom of *Trimeresurus flavoviridis. J. Biol. Chem*. **265**, 16068-16073.

Takeya, H., Nishida, S., Nishino, N., Makinose, Y., Omori-Satoh, T., Nikai, T., Sugihara, H. and Iwanaga, S. (1993) Primary structures of platelet aggregation inhibitors (disintegrins) autoproteolytically released from snake venom hemorrhagic metalloproteinases and new fluorogenic peptide substrates for these enzymes. *J. Biochem*. **113**, 473-483.

Tan, N.H. and Saifudin, M.N. (1990) Comparative study of the edema-inducing activity of snake venoms. *Comp. Biochem. Physiol*. **97C**, 293-296.

Tan, N.H, Saifuddin, M.N. and Yong, W.Y. (1991) The edema inducing activity of phospholipase A_2 enzymes. *Biochem. Int*. **23**, 175-181.

Tanaka, Y., Adams, D.H. and Shaw, S. (1993) Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol. Today* **14**, 111-115.

Teng, C.M., Wang, J.P., Peng, H.C. and Ouyang, C. (1989) Edema-producing proteins isolated from *Trimeresurus mucrosquamatus* snake venom. *Toxicon* **27**, 899-905.

Teng, C.M., Hsu, M.F. and Wang, J.P. (1992) Comparison of kinin-forming and amidolytic activities of four trimucases, oedema-producing and kinin-releasing enzymes, from *Trimeresurus mucroscquamatus* venom. *J. Pharm. Pharmacol*. **44**, 306-310.

Than, T., Soe, S. and Han, K.E. (1989) Effects of heparin on Russell's viper venom envenomated experimental rabbits. *Myanmar Hlth. Sci. Res. J.* **1**, 139-143.

Theakston, R.D.G. and Reid, H.A. (1983) Development of simple standard assay procedures for the characterization of snake venoms. *Bull. W. H. O.* **61**, 949-956.

Theakston, R.D.G. and Warrell, D.A. (1991) Antivenoms: a list of hyperimmune sera currently available for the treatment of envenoming by bites and stings. *Toxicon* **29**, 1419-1470.

Tibballs, J. and Sutherland, S.K. (1992) The efficacy of heparin in the treatment of common brown snake (*Pseudonaja textilis*) envenomation. *Anaesth. Intens. Care* **20**, 33-37.

Tilg, H., Vannier, E., Vachino, G., Dinarello, C.A. and Mier, J.W. (1993) Antiinflammatory properties of hepatic acute phase proteins: preferential induction of interleukin 1 (IL-1) receptor antagonist over IL-1β synthesis by human peripheral blood mononuclear cells. *J. Exp. Med.* **178**, 1629-1636.

Tilmisany, A.K., Mustafa, A.A., Abdel-Aziz, A. and Osman, O.H. (1986) Evidence for the presence of histamine in Gaboon viper (*Bitis gabonica*) venom. *Toxicon* **24**, 1159-1161.

Timpl, R. (1989) Structure and biological activity of basement membrane proteins. *Eur. J. Biochem*. **180**, 487-502.

Tinoco, R.C. (1972) Picada de cobra: uso de heparina como substituto de soro antiofídico. *Jorn. Bras. Med*. **23**, 109-114.

Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350.

Trebien, H.A. and Calixto, J.B. (1989) Pharmacological evaluation of rat paw edema induced by *Bothrops jararaca* venom. *Agents Actions* **26**, 292-300.

Trevors, J.T. (1986) A BASIC program for estimating LD50 values using the IBM-PC. *Bull. Environ. Contam. Toxicol*. **37**, 18-26.

Trowbridge, H.O. and Emling, R.C. (1989) *Inflammation: a Review of the Process*, 166 pp. Chicago, Quintessence Publishing.

Tu, A.T. (1977) *Venoms: Chemistry and Molecular Biology*, 560 pp. New York, Wiley and Sons.

Tu, A.T. and Homma, M. (1970) Toxicologic study of snake venoms from Costa Rica. *Toxicol. Appl. Pharmacol*. **16**, 73-78.

Tu, A.T. and Morita, M. (1983) Attachment of rattlesnake venom myotoxin *a* to sarcoplasmic reticulum: peroxidase conjugated method. *Br. J. Exp. Pathol*. **64**, 633-637.

Utaisinchareon, P., Baker, B., and Tu, A.T. (1991) Binding of myotoxin *a* to sarcoplasmic reticulum Ca^{2+}-ATPase: a structural study. *Biochemistry* **30**, 8211-8216.

Vaccari, F. and Fregnan, L. (1955) Effetto degli anticoagulanti sull'emolisi da lisine semplici. *Progr. Med. Napoli* **11**, 595-598.

Valerio, R.M., Bray, A.M., Campbell, R.A., DiPasquale, A., Margellis, C., Rodda, S.J., Geysen, H.M. and Maeji, N.J. (1993) Multipin peptide synthesis at the micromole scale using 2-hydroxyethyl methacrylate grafted polyethylene supports. *Int. J. Peptide Protein Res*. **42**, 1-9.

van den Bergh, C.J., Slotboom, A.J., Verheij, H.M. and de Haas, G.H. (1989) The role of Asp-49 and other conserved amino acids in phospholipases A_2 and their importance for enzymatic activity. *J. Cell. Biochem*. **39**, 379-390.

Vandekerckhove, F., Opdenakker, G., Van Ranst, M., Lenaerts, J.P., Put, W., Billiau, A. and Van Damme, J. (1991) Bradykinin induces interleukin-6 and synergizes with interleukin-1. *Lympokine Cytokine Res.* **10**, 285-289..

Van Loveren, H., Kato, K., Ratzlaff, R.E., Meade, R., Ptak, W. and Askenase, P.W. (1984) Use of micrometers and calipers to measure various components of delayed-type hypersensitivity ear swelling reactions in mice. *J. Immunol. Meth.* **67**, 311-319.

Van Snick, J. (1990) Interleukin-6: an overview. *Annu. Rev. Immunol*. **8**, 253-278.

Vargaftig, B.B., Bhargava, N. and Bonta, I.L. (1974) Haemorrhagic and permeability increasing effects of *Bothrops jararaca* and other Crotalidae venoms as related to amine or kinin release. *Agents Actions* **4**, 163-168.

Vishwanath, B.S., Kini, R.M. and Gowda, T.V. (1985) Purification of an edema-inducing phospholipase A_2 from *Vipera russelli* venom and its interaction with aristolochic acid. *Toxicon* **23**, 617.

Vishwanath, B.S., Kini, R.M. and Gowda, T.V. (1987) Characterization of three edema-inducing phospholipase A_2 enzymes from Habu (*Trimeresurus flavoviridis*) venom and their interaction with the alkaloid aristolochic acid. *Toxicon* **25**, 501-515.

Vishwanath, B.S., Kini, R.M. and Gowda, T.V. (1988) Purification and partial biochemical characterization of an edema inducing phospholipase A_2 from *Vipera russelli* (Russell's viper) snake venom. *Toxicon* **26**, 713-720.

Vogel, C.W. (1991) Cobra venom factor: the complement-activating protein of cobra venom. In: *Reptile Venoms and Toxins, Handbook of Natural Toxins* (Tu, A.T., Ed.), vol.5, pp.147-188. New York, Marcel Dekker Inc.

Vogt, W. (1990) Snake venom constituents affecting the complement system. In: *Medical Use of Snake Venom Proteins* (Stocker, K., Ed.), pp.79-96. Boca Raton, CRC Press.

Volpe, P., Damiani, E., Maurer, A. and Tu, A.T. (1986) Interaction of myotoxin *a* with the Ca^{2+}-ATPase of skeletal muscle sarcoplasmic reticulum. *Archs. Biochem. Biophys*. **246**, 90-97.

Wang, J.P. and Teng, C.M. (1990*a*) Comparison of the enzymatic and edema-producing activities of two venom phospholipase A_2 enzymes. *Eur. J. Pharmacol*. **190**, 347-354.

Wang, J.P. and Teng, C.M. (1990*b*) Rat paw oedema and mast cell degranulation caused by two phospholipase A_2 enzymes isolated from *Trimeresurus mucrosquamatus* venom. *J. Pharm. Pharmacol*. **42**, 846-850.

Wang, J.P. and Teng, C.M. (1991) Effects of anti-inflammatory drugs on rat hind-paw swelling caused by phospholipase A_2 from *Naja naja atra* venom. *Naunyn-Schmiedeberg's Archs. Pharmacol*. **344**, 377-381.

Wang, J.P. and Teng, C.M. (1992) Roles of PMN leucocytes, platelets and some mediators in rat hind-paw oedema induced by two phospholipase A_2 enzymes from *Trimeresurus mucrosquamatus* venom. *J. Pharm. Pharmacol*. **44**, 300-305.

Wang, J.P., Peng, H.C. and Teng, C.M. (1991) Rat hind-paw swelling effect of an edema-producing protein isolated from *Trimeresurus mucrosquamatus* snake venom. *Naunyn-Schmiedeberg's Archs. Pharmacol.* **343**, 399-404.

Warrell, D.A. (1993) Snake bite and snake venoms (Editorial). *Quart. J. Med*. **86**, 351-352.

Warrell, D.A. and Fenner, P.J. (1993) Venomous bites and stings. *Br. Med. Bull*. **49**, 423-439.

Warrell, D.A., Pope, H.M. and Prentice, C.R.M. (1976*a*) Disseminated intravascular coagulation caused by the carpet viper (*Echis carinatus*): trial of heparin. *Br. J. Haematol.* **33**, 335-342.

Warrell, D.A., Greenwood, B.M., Davidson, N.McD., Ormerod, L.D. and Prentice, C.R.M. (1976*b*) Necrosis, haemorrhage and complement depletion following bites by the spitting cobra (*Naja nigricollis*). *Quart. J. Med.* **45**, 1-22.

Warrell, D.A. (1980) Comparison of Pasteur and Behringwerke antivenoms in envenoming by the carpet viper (*Echis carinatus*). *Br. Med. J.* **1**, 607-609.

Weiss, H.J., Phillips, L.L., Hopewell, W.S., Phillips, G., Christy, N.P. and Nitti, J.F. (1972) Heparin therapy in a patient bitten by a saw-scaled viper (*Echis carinatus*), a snake whose venom activates prothrombin. *Am. J. Med.* **54**, 653-662.

Williams, G.T. and Smith, C.A. (1993) Molecular regulation of apoptosis: genetic controls on cell death. *Cell* **74**, 777-779.

Whittaker, V.P. (1990) The contribution of drugs and toxins to understanding cholinergic function. *Trends Pharmacol. Sci.* **11**, 8-19.

World Health Organization (1981) Progress in the characterization of venoms and standardization of antivenoms. Geneva, W.H.O. Offset Publication N°58.

WuDunn, D. and Spear, P.G. (1989) Initial interaction of Herpes Simplex virus with cells is binding to heparan sulfate. *J. Virol.* **63**, 52-58.

Yagihashi, S., Nikai, T., Mori, N., Kishida, M. and Sugihara, H. (1986) Characterization of Ac3-proteinase from the venom of *Agkistrodon acutus* (hundred-pace snake). *Int. J. Biochem.* **18**, 885-892.

Yamakawa, M., Nozaki, M. and Hokama, Z. (1976) Fractionation of Sakishima-habu (*Trimeresurus elegans*) venom and lethal, hemorrhagic and edema-forming activities of the fractions. In: *Animal, Plant and Microbial Toxins* (Ohsaka, A., Hayashi, K. and Sawai, Y., Eds.), vol.1, p.97. New York, Plenum Press.

Yoshizumi, K., Liu, S.Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S. and Kihara, H. (1990) Purification and amino acid sequence of basic protein I, a lysine-49 phospholipase A_2 with low activity, from the venom of *Trimeresurus flavoviridis* (Habu snake). *Toxicon* **28**, 43-54.

Yurchenco, P.D. and Schittny, J.C. (1990) Molecular architecture of basement membranes. *FASEB J.* **4**, 1577-1590.

Zhou, F., Höök, T., Thompson, J.A. and Höök, M. (1992) Heparin protein interactions. In: *Heparin and Related Polysaccharides* (Lane, D.A., Björk, I. and Lindahl, U., Eds), pp.141-153. New York, Plenum Press.

Ziolkowski C. and Bieber, A.L. (1992) Mojave toxin affects fusion of myoblasts and viability of myotubes in cell cultures. *Toxicon* **30**, 733-744.

I

Inflammation, Vol. 17, No. 2, 1993

HOST RESPONSE TO *Bothrops asper* SNAKE VENOM
Analysis of Edema Formation, Inflammatory Cells, and Cytokine Release in a Mouse Model

BRUNO LOMONTE,[1,2] ANDREJ TARKOWSKI,[2,3] and LARS Å. HANSON[2]

[1]*Instituto Clodomiro Picado, Facultad de Microbiología Universidad de Costa Rica, San José, Costa Rica*

[2]*Department of Clinical Immunology, University of Göteborg Göteborg, Sweden*

[3]*Department of Rheumatology, University of Göteborg Göteborg, Sweden*

Abstract—As part of the characterization of the host reactivity to the venom of *Bothrops asper*, we investigated the inflammatory responses in the mouse footpad model. The subcutaneously injected venom induced a rapid increase of serum IL-6 concentration, which peaked between 3 and 6 h and returned to normal values at 12 h. In contrast, serum TNF-α and IL-1α were not detectable at any time point studied. A myotoxic phospholipase A_2 isoform purified from this venom, myotoxin II, was also able to induce a systemic IL-6 release when injected into the footpad. Both venom and myotoxin induced local edema and a leukocyte infiltrate accumulating in the muscle and subdermal tissue within 6 h. The infiltrate consisted predominantly of neutrophils at 6 and 24 h, but at later times, mononuclear cells also appeared. The edema, leukocyte infiltration, and IL-6 responses did not depend on the hemorrhagic activity of venom, since all three effects were seen after injection of (1) preneutralized venom, devoid of hemorrhagic activity, and (2) purified myotoxin II. Circulating platelet numbers were significantly decreased 30 min after venom injection and returned to normal after 12 h. The venom also induced a rapid inversion in the ratio of neutrophils to lymphocytes in peripheral blood, which did not normalize until 12 h later. The present observations suggest that venom, besides its cytotoxic properties, induces early hematologic and immunologic alterations. These findings may be of relevance in future treatment modalities.

INTRODUCTION

Inflammation is a main characteristic of snakebite poisoning by viperid and crotalid species (1, 2). In Latin America, the majority of snakebites are caused

0360-3997/93/0400-0093$07.00/0

by species classified into the genus *Bothrops* (3, 4). Venoms from these snakes induce a prominent local edema both in humans and experimental animals (1, 3, 5), which, besides being responsible for significant fluid loss, can indirectly contribute to other detrimental effects of venom, by causing tissue compression and ischemia. It has been shown that the edema-inducing activity of many snake venoms is not well neutralized by conventional antivenoms, even if these are preincubated with venom prior to injection into animals (5–7). In addition to edema, a considerable local inflammatory cell response is also observed after injection of snake venoms (8, 9). A better understanding of the inflammatory events that follow envenomation could lead to the development of better therapeutic strategies, complementary to conventional serotherapy. Moreover, venoms and isolated toxins may be utilized as tools for the study of basic inflammatory mechanisms (10).

As part of the characterization of the inflammatory response to the venom of *B. asper*, we investigated in the present work the formation of edema, the blood and tissue changes of inflammatory cells, and the release of cytokines in the mouse footpad model.

MATERIALS AND METHODS

Venom, Antivenom, and Myotoxin II. Venom obtained from more than 30 specimens of *B. asper* from the Pacific region of Costa Rica was pooled, lyophilized, and stored at −20°C (batch BAP-9-83). The equine polyvalent antivenom produced at Instituto Clodomiro Picado, Universidad de Costa Rica (batch P-183), as described by Bolaños and Cerdas (11), was utilized in neutralization experiments. Myotoxin II was isolated from the venom by CM-Sephadex chromatography as described by Lomonte and Gutiérrez (12).

Edema-Forming Activity. Edema was quantified in the footpad of mice (SWISS, 22–28 g) by measuring the increase of thickness with a low-pressure spring caliper (Oditest, H. C. Kröplin, Germany) (13). In order to facilitate measurements, mice were anesthesized by ether inhalation or by intraperitoneal injection of Mebumal (NordVacc, Sweden; 0.6 mg/10 g body weight).

Selection of Venom Dose. Groups of five mice received a subcutaneous injection of venom in the left footpad, dissolved in 50 μl of phosphate-buffered saline, pH 7.2 (PBS). The venom doses were 1, 10, 25, 50, 100, and 1000 μg. Edema was quantified at 0, 0.5, 1, 3, 6, 12, 24, 48, and 72 h after the injection.

Histological Analysis. Groups of three mice received a subcutaneous injection of either 1 or 50 μg of venom in the left footpad. Animals were sacrificed by cervical dislocation at 15, 30 min, 1.5, 6, 24, 72 h, and one week after injection. The injected limb was cut, fixed in 4% paraformaldehyde, 0.1 M cacodylate buffer (pH 7.4), decalcified, and embedded in paraffin. Sections were stained with hematoxylin–eosin.

Hematological Evaluation. Venom (50 μg) was injected subcutaneously into the footpad of mice and at 0, 1, 3, 6, 12, 24, 72 h, and 7 days; heparinized (200 units/ml) blood samples were obtained under anesthesia by cutting the brachial artery. White blood cells and platelets were counted in a Sysmex analyzer (K-1000, Toa Medical Electronics, Japan). Differential counts for leukocytes were performed on Giemsa-stained blood smears.

Quantification of Tumor Necrosis Factor-α (TNF-α). A cytotoxicity assay using the WEHI

164 clone 13 murine fibrosarcoma cell line (14) was utilized. Cells were cultured in Iscove's medium (Gibco, Paisley, U.K.) supplemented with 10% fetal calf serum (Biological Ind., Haemek, Israel), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 0.05 mg/ml gentamycin, at $2–4 \times 10^4$ cells/well. Cultures were performed in 96-well plates at 37°C in 5% CO_2, in the presence of either test samples (diluted 1:5 and 1:25 with medium) or recombinant murine TNF-α standards (Genzyme, Cambridge, Massachusetts), ranging from 0.06 to 1000 pg/ml. After 20 h, 10 μl/well of a 5 mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, Missouri) were added for 4 h. Then, the medium was decanted and the formazan was dissolved with 100 μl/well of 95% ethanol for 10 min, with constant shaking. Absorbance was read at 578 nm on a Titertek Multiskan (Flow Laboratories, Finland). Standards were diluted with medium in the case of assays of cell culture supernatants, or with normal mouse serum at a comparable final concentration in assays of mouse sera. Standards and samples were assayed in triplicate. The lower detection limit was 0.1 pg/ml.

Quantification of Interleukin-6 (IL-6). The murine B-cell hybridoma line B9 was utilized (15). Cells were maintained in Iscove's medium as described above, supplemented with recombinant murine IL-6 (8 pg/ml; AMS Biotechnology, Sweden). At the moment of the assay, cells were harvested, washed twice with medium, and cultured in 96-well plates at 5×10^3 cells/well in the presence of either test samples (diluted 1:50 and 1:250 with medium) or IL-6 standards ranging from 0.12 to 8 pg/ml, during 72 h. Then, 1 μCi/well of [^{3}H]thymidine (Amersham, U.K.) was added, and after 4 h cells were harvested and the incorporation of thymidine was measured on a direct β-counter (Inotech, Switzerland). Standards and samples were run in triplicate.

Quantification of Interleukin-1α (IL-1α). A monoclonal antibody-based enzyme-immunoassay (Intertest 1α-X, Genzyme) was utilized. The lower detection limit was 15 pg/ml. Sera were tested at a dilution of 1:4, according to the manufacturer's instructions.

Release of IL-6, TNF-α, and IL-1α In Vivo. Groups of four mice received a subcutaneous injection of venom (1 or 50 μg) in the footpad. Control mice received a PBS injection. After 1, 3, 6, 12, 24, 72 h, and 7 days, mice were bled, and levels of IL-6, IL-1α, and TNF-α were quantified in serum.

Effects of Venom on Spleen Cells In Vitro

Mitogenicity. A spleen cell suspension obtained from a single mouse was cultured in 96-well plates at 4×10^5 cells/well (16) in the presence of venom (1, 10, 100 pg/ml; 1, 10, 100 ng/ml; 1 or 10 μg/ml) during 72 h. Venom was sterilized by filtration through a 0.22-μm membrane. As a positive control, *E. coli* B 055:B5 lipopolysaccharide (LSP; 10 μg/ml; Difco, Detroit, Michigan) was included. Cell proliferation was quantified by incorporation of [^{3}H]-thymidine.

Cytotoxicity. Spleen cells were cultured in 24-well plates at 2×10^6 cells/well in the presence of venom (1, 10, 100 pg/ml; 1, 10, 100 ng/ml; 1 or 10 μg/ml). After 6, 24, 48, and 72 h, cells were harvested and viability was estimated by an automated cell counter (Sysmex F-300, Toa Medical Electronics, Japan).

Release of IL-6 and TNF-α by Spleen Cells In Vitro. The supernatants from the cultures utilized for testing cytotoxicity were collected after 6, 24, 48, and 72 h and assayed for the presence of TNF-α and IL-6 as described above.

Neutralization of Venom by Antivenom. Groups of four mice received subcutaneous injection in the footpad of a mixture of antivenom and venom (ratio 0.75 ml antivenom/mg venom) preincubated at 37°C for 30 min. A control group received only venom (50 μg). Edema was measured at 0, 1, 3, and 6 h, and blood samples for the quantification of serum IL-6 were collected at 3 and 6 h after the injection. Footpad tissue obtained at 6 h was processed for histological evaluation.

Induction of IL-6 by Purified Myotoxin II. Myotoxin II (100 μg) was injected subcutaneously in the footpad of groups of four mice, and edema was measured at 0, 1, 3, and 6 h. Blood samples for IL-6 quantification were collected at 3 and 6 h, and tissue samples were obtained at 6 h. Control mice received 50 μl of PBS.

Statistical Analysis. The two-tailed Student's *t* test was utilized to evaluate the differences between means.

RESULTS

Edema-Forming Activity. The time course of edema induced by different venom doses was followed during a 72-h period (Figure 1). The footpad thickness increased rapidly after injection. At 0.5 and 1 h, a similar degree of edema was obtained with all doses tested. However, after 1 h, the profile observed with 1 μg was markedly different from the other doses, decreasing rapidly to normal (Figure 1). In contrast to the other doses, 1 μg did not induce macroscopically observable hemorrhage. The mice receiving the highest dose of venom (1000 μg) died within 12 h after injection, all the lower doses being sublethal. Injection of 100 μg induced local skin ulceration, which caused loss of some edema fluid. Based on these results, the venom dose selected for the study of inflammation was 50 μg. The dose of 1 μg was also included in several experiments, since it induced a marked but transient edema, in the apparent absence of hemorrhage.

Histological Analysis. Histologically, a marked subcutaneous edema was evident as early as 15 min after the injection of both 1 and 50 μg venom doses. However, hemorrhage was seen only with the 50-μg dose, both in the muscle and subcutaneous layers. At 72 h, only few foci of extravasated red blood cells

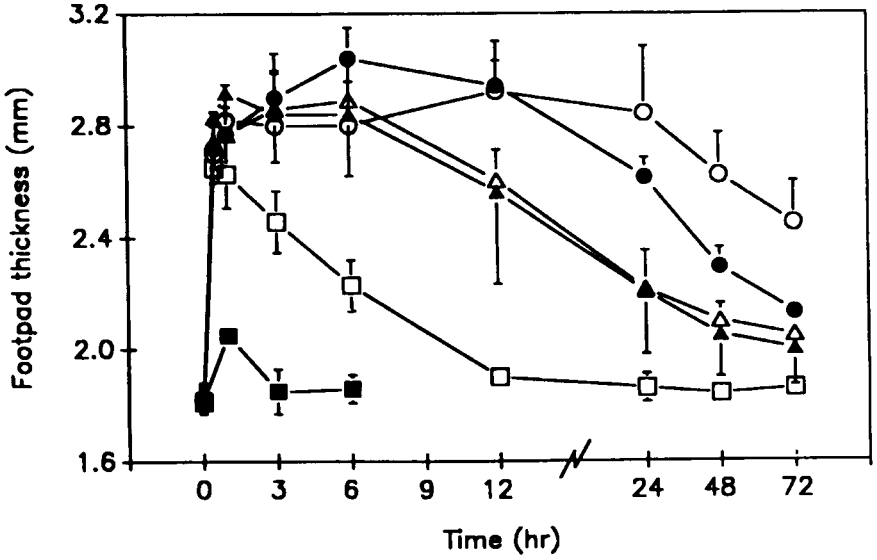

Fig. 1. Time course of the edema-forming activity of *B. asper* venom injected subcutaneously in the footpad of mice. Groups of five mice received a subcutaneous venom injection in the footpad in a total volume of 50 μl. The footpad thickness was measured with a low-pressure spring caliper at the times indicated. Each point represents mean ± *SD*. ○, 100 μg; ●, 50 μg; △, 25 μg; ▲ 10 μg; □, 1 μg; ■, phosphate-buffered saline.

remained, despite the extensive initial hemorrhage. A diffuse inflammatory cellular infiltrate accumulated in the subcutaneous and muscle layers 6 h after injection, predominantly consisting of polymorphonuclear neutrophils (Figure 2). The infiltrate elicited by 1 μg of venom was very scarce in comparison to that observed with 50 μg injection, at all time points studied. At 24 and 72 h, the cellular infiltrate increased, and mononuclear cells, with morphologic characteristics of macrophages, were observed in addition to neutrophils. After one week, the cellular infiltrate had almost disappeared, and some isolated foci of fibrotic tissue containing macrophages were seen.

Hematological Changes. A significant decrease in the number of circulating platelets was observed 30 min after envenomation (Figure 3). This decrease lasted for 12 h. On the other hand, a moderate but significant ($P < 0.05$) increase in leukocyte counts was found after 12 h and lasted up to one week (Figure 4). Even though the total white blood cell numbers did not change significantly during the first 6 h, a rapid inversion of the proportion of lymphocytes to neutrophils was observed in this time period, with neutrophils increasing significantly ($P < 0.05$) at 1, 3, and 6 h. The lymphocyte–neutrophil ratio normalized within 24 h (Figure 4).

Changes in Serum Cytokine Levels. The venom induced a rapid elevation of serum IL-6, which peaked between 3 and 6 h after the injection of 50 μg, and returned to normal values at 12 h (Figure 5). In repeated experiments, the peak concentration varied between 60 and 120 pg/ml, representing 10- to 15-fold increase. Injection of PBS did not increase IL-6 levels. Only a slight increase in serum IL-6 concentration was observed after injection of 1 μg of venom. No detectable serum levels of TNF-α or IL-1α were observed after the injection of 50 μg of venom (data not shown). As a control, sera obtained from mice injected with *E. coli* LPS were found to contain high levels of both TNF-α and IL-1α.

Effects of Venom on Spleen Cells In Vitro. The venom did not have a direct mitogenic effect on murine spleen cells when tested over a wide range of concentrations (data not shown). In contrast, LPS used as a control had a strong mitogenic activity. The venom displayed in vitro cytotoxicity to spleen cells in a dose- and time-dependent manner (Figure 6). Cytotoxicity was higher in the microgram range of concentrations and negligible at concentrations below 10 pg/ml. However, no significant venom-induced release of either IL-6 or TNF-α was detected in the supernatants at any time tested or any venom concentration (not shown). In contrast, supernatant from LPS-containing cultures showed high concentrations of both cytokines. Control experiments indicated that the venom did not have direct effects on the indicator systems utilized for the cytokine bioassays; neither changed the activity of recombinant TNF-α or IL-6 when these were preincubated with the venom prior to the assays.

Neutralization by Antivenom. The antivenom did not reduce the extent of edema induced by the venom (Figure 7A). However, both macroscopically and

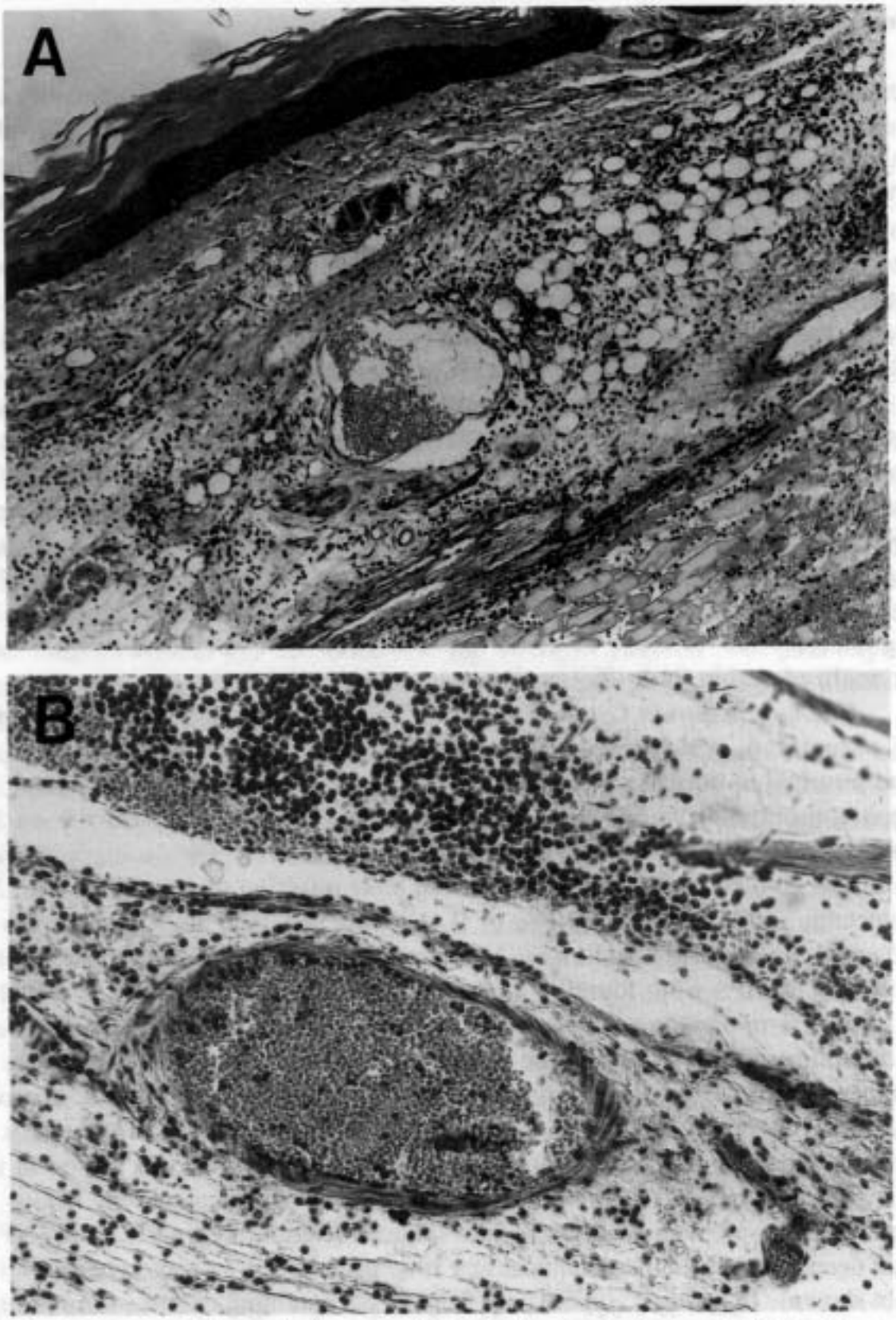

Fig. 2. Inflammatory infiltrate in the mouse footpad after a subcutaneous injection of *B. asper* venom. (A) Hemotoxylin–eosin stained histologic section of the footpad, 24 h after the subcutaneous injection of 50 μg of *B. asper* venom. A dispersed leukocyte infiltrate is seen (predominantly polymorphonuclear neutrophils), together with extravasated red blood cells and an area of myonecrosis. Magnification $\times 32$. (B) Same as above, 6 h after injection. Note the increased adherence of leukocytes to the walls of a vein and the extravascular accumulation of leukocytes. Magnification $\times 64$.

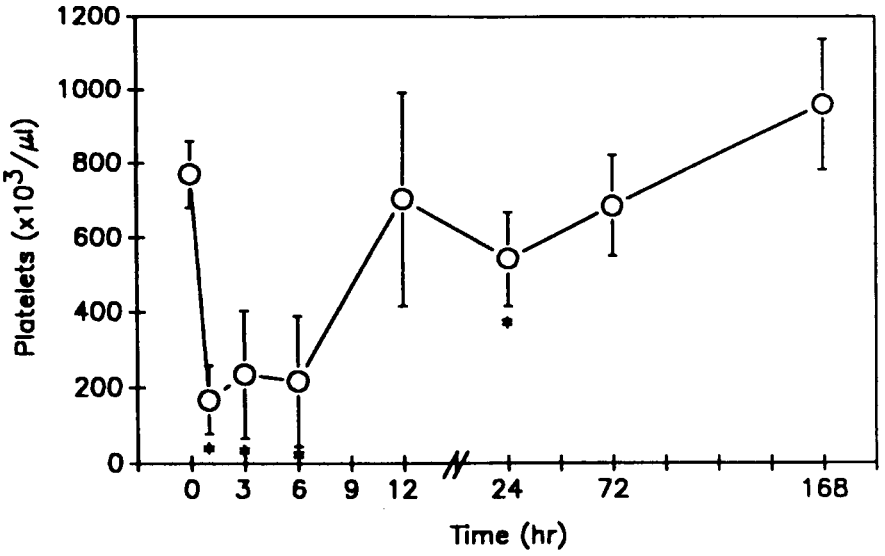

Fig. 3. Changes in numbers of circulating platelets after a subcutaneous injection of *B. asper* venom. Groups of four mice received subcutaneous venom injection in the footpad (50 μg) and blood platelet numbers were determined at the time points indicated. Each point represents mean ± *SD*. Asterisks below the values indicate a statistically significant ($P < 0.05$) difference as compared to time 0.

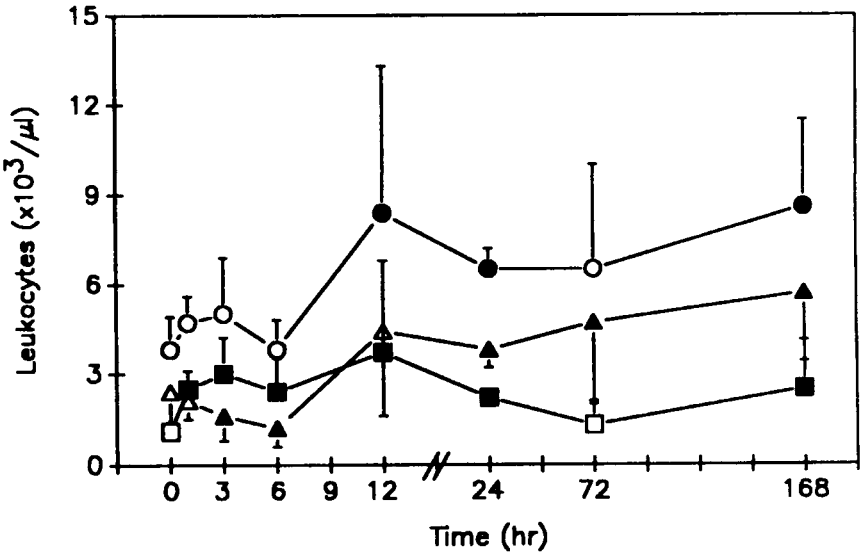

Fig. 4. Changes in numbers of circulating leukocytes after subcutaneous injection of *B. asper* venom. Groups of four mice received a subcutaneous venom injection in the footpad (50 μg) and total white blood cells (○), lymphocytes (△), and neutrophils (□) were determined at the time points indicated. Each point represents mean ± *SD*. Filled symbols indicate a statistically significant ($P < 0.05$) difference as compared to time 0.

histologically, the hemorrhage was completely neutralized by the antivenom. Interestingly, an abundant polymorphonuclear infiltrate was still observed. The antivenom partially reduced the release of IL-6 at both 3 and 6 h in comparison to the group receiving venom only ($P < 0.05$; Figure 7B).

Induction of Serum IL-6 by Myotoxin II. Myotoxin II induced a rapid

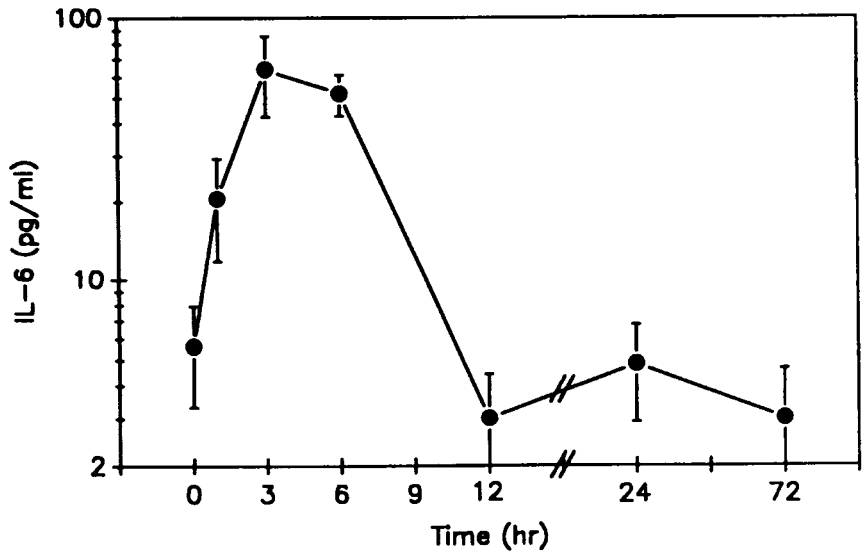

Fig. 5. Induction of interleukin-6 production after subcutaneous injection of *B. asper* venom. Groups of four mice received a subcutaneous injection of 50 μg of venom in the footpad and were bled at the indicated time points. IL-6 was quantified by bioassay as described in Materials and Methods. Each point represents mean ± *SD*. Values corresponding to one week after injection were normal (<5 pg/ml) and were omitted from the figure to enhance the resolution of the time scale.

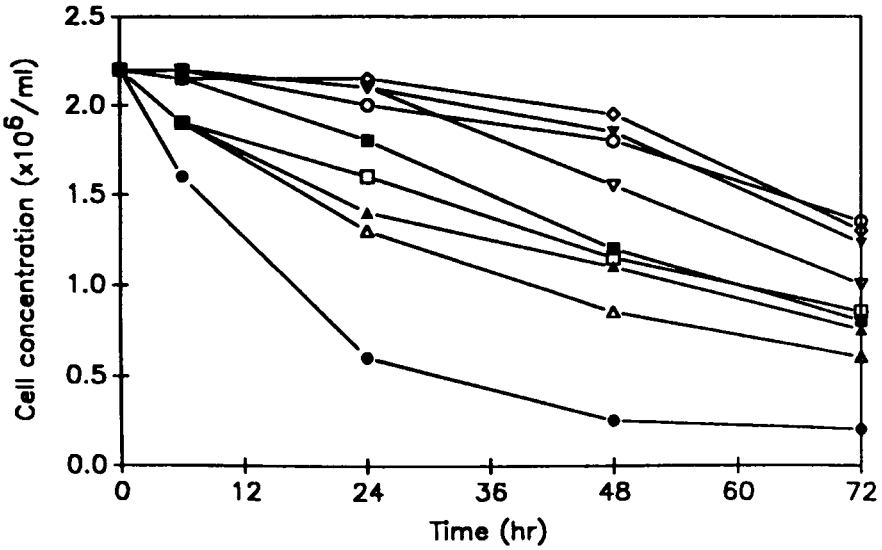

Fig. 6. Cytotoxic activity of *B. asper* venom on murine spleen cells in vitro. Spleen cells were cultured in the presence of variable concentrations of venom and then the residual viable cells were counted at the time points indicated. Each point represents the mean of duplicate determinations. Variability is smaller than symbol size. ♦, medium only; ●, 10 μg/ml; △, 1 μg/ml; ▲, 100 ng/ml; □, 10 ng/ml; ■, 1 ng/ml; ▽, 100 pg/ml; ▼, 10 pg/ml; ◇, 1 pg/ml.

edema peak after the injection, which was maintained during the 6-h observation period (Figure 7A). The toxin also induced a marked IL-6 increase both at 3 and 6 h after injection (Figure 7B). A moderate polymorphonuclear cell infiltrate was observed histologically.

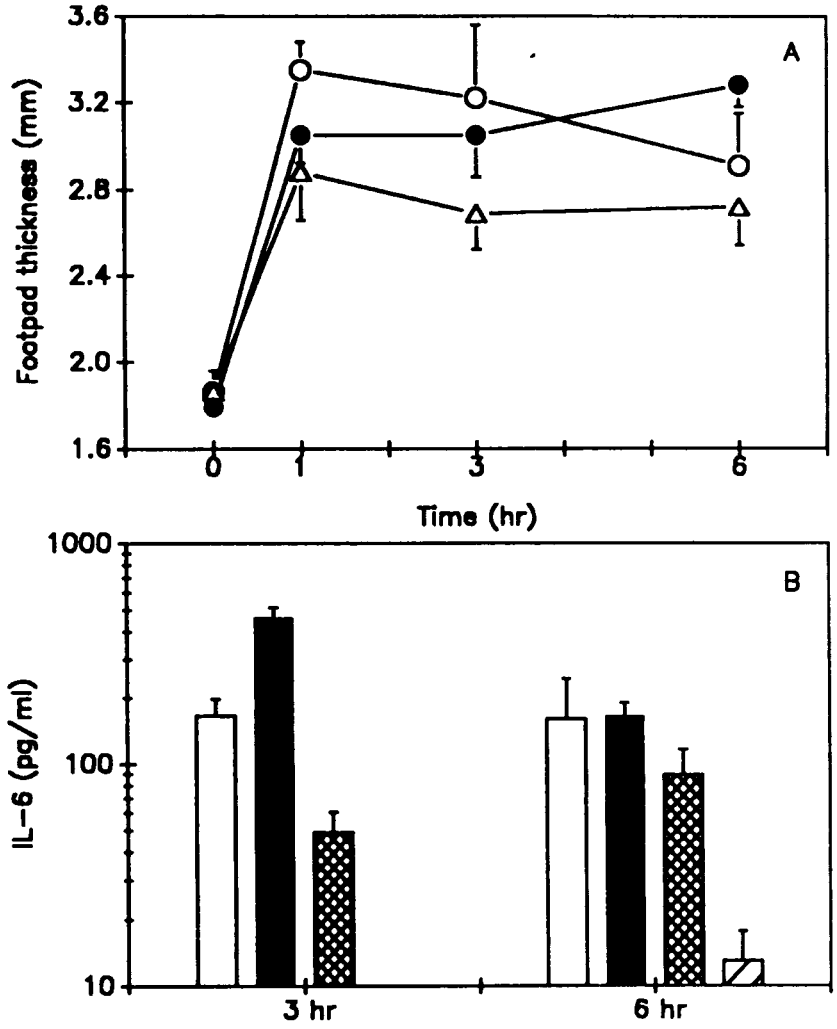

Fig. 7. Induction of edema and interleukin-6 production by *B. asper* venom after preincubation with antivenom and by *B. asper* myotoxin II in mice. (A) Edema induced by 50 μg of venom (●) venom preincubated with antivenom (○), or 100 μg myotoxin II (△) injected subcutaneously in the footpad of mice ($N = 4$). Each point represents mean ±SD. There are no statistically significant ($P > 0.05$) differences between the effects of venom and preneutralized venom. (B) Interleukin-6 concentration in the serum of mice from the experiment described in (A). Bars represent mean ± *SD*. Empty bars: venom; filled bars: myotoxin II; cross-hatched bars: preneutralized venom; wide hatched bar: phosphate-buffered saline. Statistical differences are presented in Results.

DISCUSSION

The local tissue-damaging effects of *B. asper* venom include hemorrhage, myonecrosis, and edema (8). Two types of edema responses to the venom were observed in the present study: a rapid and transient reaction, induced by 1 μg, with no signs of hemorrhage, and a rapid but long-lasting reaction, induced by higher doses, which included severe hemorrhage. Thus, the edema-inducing effect of this venom is probably due to several types of components acting in combination (17). However, the nature of the factor(s) involved in this potent

activity is unknown. Present results are in agreement with those of Tan and Saifuddin (18), showing that the edema induced by a variety of venoms peaked 1 h after injection and declined thereafter if low doses, below the threshold for hemorrhage, were utilized. While the use of a low dose appears to be important for an accurate estimation of the edema-inducing potency of snake venoms (18), higher doses capable of reproducing the pathological picture observed after snakebites seem to be more appropriate for the study of inflammatory events. Indeed, at the histological level, a very scarce inflammatory infiltrate was observed after the injection of 1 μg, despite the marked edema. In contrast, after the injection of 50 μg, an abundant leukocyte infiltrate accumulated in the footpad tissue within 6 h, increasing at 24 and 72 h, and declining thereafter. This inflammatory response was described after injection of *B. asper* venom into skeletal muscle (8). It has been suggested that leukocyte-derived enzymes may play a role in the degradation of myofibrillar proteins after venom-induced necrosis (9, 19). The kinetics and cell composition of the inflammatory infiltrate that developed in the footpad appeared to be similar to those previously observed in muscle (9).

Regarding leukocyte changes in blood, there was a rapid inversion of the lymphocyte–neutrophil ratio after venom injection. The ratio normalized after 12 h, but then a moderate increase of leukocyte numbers was observed. Results agree with those of Barrantes et al. (20), reporting increased leukocyte numbers in patients envenomed by *B. asper*. However, in the same study, no significant changes in platelet numbers were found, despite marked alterations in the coagulation system (20). This contrasts with the present findings in mice, where a marked decrease in circulating platelets was observed. The difference could be due to species variations, but also due to sample timing, since measurements in the clinical study of Barrantes et al. (20) were performed at unspecified times varying between 2 and 24 h after the snakebite, and, at least in our model, platelet numbers were already normal by 12 h. The rapid production of IL-6 observed after envenomation could play a role in the normalization of platelet numbers, since this cytokine increases platelet numbers in mice and monkeys (21).

The sequence of events leading to the local accumulation of leukocytes after venom injection is not known, and, due to the complex nature of venoms in terms of pharmacologically active components, many possibilities exist. We investigated the involvement of cytokines, particularly TNF-α, IL-1α, and IL-6, which are known to participate in a variety of inflammatory conditions (21–23). In serum, a significant increase of IL-6 levels was observed soon after venom injection, while no IL-1α or TNF-α increases were detected. The possibility of local production of IL-1α and TNF-α (24) was not ruled out and is presently being evaluated by immunohistochemical techniques. Both T and B

lymphocytes have been identified as sources of IL-6 (21), as have macrophages (25) and many other cell types. However, in vitro incubation of venom with mouse spleen cells did not lead to cytokine release or mitogenic stimulation, despite the significant dose-dependent cytotoxicity observed. This indicates that lymphocytes and macrophages are probably not responsible for the in vivo production of IL-6 after envenomation. Another possible source of IL-6 could be the endothelial cells (26), since the venom has drastic effects on the integrity of blood vessels. This hypothesis has to be tested directly using cultured endothelial cells. Two lines of evidence showed that the systemic IL-6 increase does not depend on the hemorrhagic action of the venom. First, preneutralized venom was devoid of hemorrhagic activity but caused a significant serum IL-6 increase. Second, purified myotoxin also induced IL-6 in vivo, in the absence of hemorrhage. In both cases a local inflammatory infiltrate appeared, indicating that infiltration also occurs independently of the hemorrhagic effect of the venom.

Since the mouth and fangs of snakes possess a bacterial flora (27), lipopolysaccharide could be a possible venom contaminant, which should be considered due to its inflammatogenic activities (22). However, the lack of mitogenic and in vitro cytokine-induction activities of the venom suggests that endotoxin, if present, does not play a significant role in this model.

The observation that purified myotoxin II was also able to induce an IL-6 response is particularly interesting. This toxin is a lysine-49 phospholipase A_2 (28). Some snake venom phospholipases A_2 share common structural features with mammalian extracellular phospholipases A_2 (29), which are important mediators of inflammation (30). It was previously shown that myotoxin II has a moderate edema-inducing activity and that injection into muscle leads to an inflammatory infiltrate appearing after myonecrosis (12). The infiltrate was also observed in the footpad model, not only among muscle fibers but also in the subdermal layer. The study of the mechanism of IL-6 induction by myotoxin could provide important clues for understanding the actions of crude venom. The present findings using pure myotoxin suggest that myonecrosis itself could be an initial stimulus for the venom-induced release of IL-6. It has been shown recently that IL-6 induces a proliferative response of myoblasts in culture (31). Thus, the release of IL-6 in response to envenomation might be an important physiological mechanism for the initiation of skeletal muscle regeneration, a process observed after both venom- and myotoxin-induced myonecrosis (32–34).

Acknowledgments—We are grateful to Lena Svensson and Ing-Marie Nilsson for excellent assistance and to Arturo Abdelnour, José María Gutiérrez and Cecilia Díaz for helpful discussions. B. Lomonte was a recipient of a research award from the Costa Rican National Scientific and Technological Research Council (CONICIT) during this study.

REFERENCES

1. ROSENFELD, G. 1971. Symptomatology, pathology and treatment of snake bites in South America. *In* Venomous Animals and Their Venoms. W. Bucherl, and E. E. Buckley, editors). Academic Press, New York. 345–403.
2. SAWAI, Y. 1980. Studies on snakebites in the Asian areas. *In* Natural Toxins. D. Eaker and T. Wadstrom, eds., Pergamon Press, Oxford. 25–32.
3. BOLAÑOS, R. 1984. Serpientes, venenos y ofidismo en Centroamérica, Editorial Universidad de Costa Rica, San José. 136 pp.
4. CARDOSO, J. L. 1985. Snakebites at hospital Vital Brazil. A study of 2908 cases. *Toxicon* **23**:558.
5. GUTIÉRREZ, J. M., G. ROJAS, B. LOMONTE, J. A. GENÉ, and L. CERDAS. 1986. Comparative study of the edema-forming activity of Costa Rican snake venoms and its neutralization by a polyvalent antivenom. *Comp. Biochem. Physiol.* **85C**:171–175.
6. LOMONTE, B. 1985. Edema-forming activity of bushmaster (*Lachesis muta stenophrys*) and Central American rattlesnake (*Crotalus durissus durissus*) venoms and neutralization by a polyvalent antivenom. *Toxicon* **23**:173–176.
7. ROJAS, G., J. M. GUTIÉRREZ, J. A. GENÉ, M. GÓMEZ, and L. CERDAS. 1987. Neutralización de las actividades tóxicas y enzimáticas de cuatro venenos de serpientes de guatemala y Honduras por el antiveneno polivalente producido en Costa Rica. *Rev. Biol. Trop.* **35**:59–67.
8. GUTIÉRREZ, J. M., O. ARROYO, and R. BOLAÑOS. 1980. Mionecrosis, hemorragia y edema inducidos por el veneno de *Bothrops asper* en ratón blanco. *Toxicon* **18**:603–610.
9. GUTIÉRREZ, J. M., F. CHAVES, and L. CERDAS. 1986. Inflammatory infiltrate in skeletal muscle injected with *Bothrops asper* venom. *Rev. Biol. Trop.* **34**:209–219.
10. BONTA, I. L., B. B. VARGAFTIG, and G. M. BOHM. 1978. Snake venoms as an experimental tool to induce and study models of micro-vessel damage. *In* Handbook of Experimental Pharmacology. C. Y. Lee, ed. Springer, Berlin. 629–683.
11. BOLAÑOS, R., and L. CERDAS. 1980. Producción y control de sueros antiofídicos en Costa Rica. *Bol. Sanit. Panam.* **88**:184–196.
12. LOMONTE, B., and J. M. GUTIÉRREZ. 1989. A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27**:725–733.
13. VAN LOVEREN, H., K. KATO, R. E. RATZLAFF, R. MEADE, W. PTAK, and P. W. ASKENASE. 1984. Use of micrometers and calipers to measure various components of delayed-type hypersensitivity ear swelling reactions in mice. *J. Immunol. Methods* **67**:311–319.
14. ESPEVIK, T., and J. NISSEN-MEYER. 1986. A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *J. Immunol. Methods* **95**:99–105.
15. NELLE, M., L. BOEIJE, and L. AARDEN. 1988. Functional discrimination between interleukin-6 and interleukin-1. *Eur. J. Immunol.* **18**:1535–1540.
16. BRADLEY, L. M. 1980. Cell proliferation. *In* Selected Methods in Cellular Immunology. B. B. Mishell and S. M. Shiigi, eds. Freeman, San Francisco. 153–172.
17. GUTIÉRREZ, J. M., and B. LOMONTE. 1989. Local tissue damage induced by *Bothrops* snake venoms. *Mem. Inst. Butantan* **51**:211–223.
18. TAN, N.-H., and M. N. SAIFUDDIN. 1990. Comparative study of the edema-inducing activity of snake venoms. *Comp. Biochem. Physiol.* **97C**:293–296.
19. GUTIÉRREZ, J. M., V. ARCE, F. BRENES, and CHAVES, F. 1990. Changes in myofibrillar components after skeletal muscle necrosis induced by a myotoxin isolated from the venom of the snake *Bothrops asper*. *Exp. Mol. Pathol.* **52**:25–36.

20. BARRANTES, A., V. SOLÍS, and R. BOLAÑOS. 1985. Alteración de los mecanismos de la coagulación en el envenenamiento por *Bothrops asper* (terciopelo). *Toxicon* **23**:399–407.
21. HIRANO, T., S. AKIRA, T. TAGA, and T. KISHIMOTO. 1990. Biological and clinical aspects of interleukin 6. *Immunol. Today* **11**:443–449.
22. MOVAT, H. Z., M. I. CYBULSKY, I. G. COLDITZ, W. CHAN, and C. A. DINARELLO. 1987. Acute inflammation in gram-negative infection: Endotoxin, interleukin 1, tumor necrosis factor, and neutrophils. *Fed. Proc.* **46**:97–104.
23. DINARELLO, C. A. 1991. The proinflammatory cytokines interleukin-1 and tumor necrosis factor and treatment of the septic shock syndrome. *J. Infect. Dis.* **163**:1177–1183.
24. REMICK, D. G. 1991. Significance of in vivo detection of tumor necrosis factor. *Lab. Invest.* **65**:259–261 (editorial).
25. MARTIN, C. A., and M. E. DORF. 1990. Interleukin-6 production by murine macrophage cell lines P388D1 and J774A: Stimulation requirements and kinetics. *Cell. Immunol.* **128**:555–568.
26. LEEUWENBERG, J. F. M., E. J. U. VON ASMUTH, T. M. A. A. JEUNHOMME, and W. A. BUURMAN. 1990. IFN-τ regulates the expression of the adhesion molecule ELAM-1 and IL-6 production by human endothelial cells in vitro. *J. Immunol.* **145**:2110–2114.
27. ARROYO, O., and R. BOLAÑOS. 1980. The bacterial flora of venoms and mouth cavities of Costa Rican snakes. *Bull. Pan Am. Health Org.* **14**:280–284.
28. FRANCIS, B., J. M. GUTIÉRREZ, LOMONTE, B., and I. KAISER. 1991. Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A_2. *Arch. Biochem. Biophys.* **284**:352–359.
29. DAVIDSON, F. F., and E. A. DENNIS. 1990. Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venom to human secreted form. *J. Mol. Evol.* **31**:228–238.
30. PRUZANSKI, W., and P. VADAS. 1991. Phospholipase A_2—a mediator between proximal and distal effectors of inflammation. *Immunol. Today* **12**:143–146.
31. AUSTIN, L., and A. W. BURGESS. 1991. Stimulation of myoblast proliferation in culture by leukaemia inhibitory factor and other cytokines. *J. Neurol. Sci.* **101**:193–197.
32. GUTIÉRREZ, J. M., C. L. OWNBY, and G. V. ODELL. 1984. Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops asper* (fer-de-lance). *Toxicon* **22**:719–731.
33. GUTIÉRREZ, J. M., F. CHAVES, E. MATA, and L. CERDAS. 1986. Skeletal muscle regeneration after myonecrosis induced by *Bothrops asper* (terciopelo) venom. *Toxicon* **24**:223–231.
34. ARCE, V., F. BRENES, and GUTIÉRREZ, J. M. 1991. Degenerative and regenerative changes in murine skeletal muscle after injection of venom from the snake *Bothrops asper*: A histochemical and immunocytochemical study. *Int. J. Exp. Pathol.* **72**:211–226.

II

Toxicon, Vol. 31, No. 6, pp. 743–753, 1993.
Printed in Great Britain.

0041-0101/93 $6.00 + .00

BIOLOGICAL AND BIOCHEMICAL ACTIVITIES OF *VIPERA BERUS* (EUROPEAN VIPER) VENOM

LEONEL CALDERÓN,[1] BRUNO LOMONTE[1,2]* JOSÉ MARÍA GUTIÉRREZ,[1] ANDREJ TARKOWSKI[2] and LARS Å. HANSON[2]

[1]Instituto Clodomiro Picado, Facultad de Microbiologia, Universidad de Costa Rica, San José, Costa Rica; and [2]Department of Clinical Immunology, Guldhedsgatan 10, University of Göteborg, S-41346 Göteborg, Sweden

(*Received* 29 *October* 1992; *accepted* 9 *December* 1992)

L. CALDERÓN, B. LOMONTE, J. M. GUTIÉRREZ, A. TARKOWSKI and L. Å. HANSON. Biological and biochemical activities of *Vipera berus* (European viper) venom. *Toxicon* **31**, 743–753, 1993.—*Vipera berus* is widely distributed throughout the northern part of Europe and Asia. Characterization of several toxic effects of its venom in the mouse, as well as of *in vitro* enzymatic activities was performed. *Vipera berus* venom displayed *in vitro* proteolytic, fibrinolytic, anticoagulant, and phospholipase A_2 activities. The i.p. LD_{50} of the venom for Swiss mice was 0.86 μg/g (95% confidence limits 0.71–1.01 μg/g). Significant local tissue-damaging effects, including edema, hemorrhage and myonecrosis, were observed. The local edema was characterized by rapid onset, reaching a maximum after 0.5–1 hr, and with dose-dependent persistence. The hemorrhagic potency was measured by a skin test, giving a minimum hemorrhagic dose value of 3.2 μg. The venom also induced a moderate local myonecrosis, evidenced by histological evaluation of injected tissue (gastrocnemius), and by biochemical parameters (increase of plasma creatine kinase activity, and decrease of muscle residual MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide)-reducing activity). Characterization of the venom by SDS-polyacrylamide gel electrophoresis revealed 10 (reduced) or 11 (unreduced) main protein bands, which were further analyzed in relation to mol. wt and relative concentration by densitometry. A rabbit antiserum to *V. berus* venom recognized all main venom bands by immunoblotting. This antiserum cross-reacted to a variable extent with several crotaline venoms, as assessed by enzyme immunoassay.

INTRODUCTION

Vipera berus (European viper, common adder, common viper) is the venomous snake with the widest geographical distribution, ranging from the British Isles throughout Europe, north to the Artic Circle through the Confederation of Independent States, including southern Siberia, and northern People's Republic of China to the Pacific coast. It is absent from Ireland, southern Spain, Italy and the Balkans (MEHRTENS, 1987). Although rarely fatal, envenomations by *V. berus* deserve medical concern (GONZALEZ, 1991). According

* Author to whom correspondence should be addressed, at the University of Göteborg.

to HABERMEHL (1981), the World Health Organization indicated about 1300 accidents caused annually by this species in Sweden, of which 12% lead to hospitalization, with a mortality rate of 0.3%.

Clinical findings described in patients envenomed by *V. berus* include pain, stinging sensation, cramps, local edema, drowsiness or confusion, acidosis, leukocytosis, hypotension, shock, vomiting, diarrhea, ecchymosis, and angioneurotic edema of the tongue and lips (HABERMEHL, 1981; STAHEL *et al.*, 1985; CEDERHOLM and LENNMARKEN, 1987). Several components have been isolated from the venom of *V. berus* and biochemically characterized (BOFFA *et al.*, 1976; SIIGUR *et al.*, 1979, 1986, 1988; SAMUEL and SIIGUR, 1990). However, very little information is available on the pathobiological effects induced by this venom in experimental animal models. In the present work, a characterization of several toxic effects of *V. berus* venom in the mouse model is presented. Also, enzymatic activities that might have a relationship to biological effects *in vivo* were determined. Finally, due to the taxonomic position of *V. berus* as a representative of the 'Old World' vipers (Viperinae), an antiserum was produced and utilized to investigate its possible antigenic relationship to venoms of a group of 'New World' pit vipers (Crotalinae).

MATERIALS AND METHODS

Venom

Venom of *V. berus* of Russian origin (batch 122F0841) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Lethal activity

The lethal potency of the venom was determined in Swiss Webster mice (16–18 g). Groups of five animals received an i.p. injection of venom in 500 μl of sterile 0.12 M NaCl, 40 mM sodium phosphate buffer, pH 7.2 (PBS). The logarithmically spaced venom doses utilized in the final estimation were 12.7, 16.8, 22.5, 30, and 40 μg. Deaths were scored after 48 hr, and the lethal dose 50% (LD_{50}) was calculated by probit analysis using the computer program described by TREVORS (1986).

Edema-inducing activity

Local edema was quantified in the mouse footpad, by measuring the thickness increase with a low-pressure spring caliper (Oditest, H.C. Kröplin, F.R.G.) as described by VAN LOVEREN *et al.* (1984). Different venom doses (2.5–20 μg) in 50 μl of PBS were injected s.c. in the footpad of groups of five mice (18–21 g), and thickness was measured after 0.5, 1, 3, 6, 9, 24, and 48 hr. Mice were anesthetized by i.p. injection of Mebumal (NordVacc, Sweden; 0.6 mg/10 g body weight).

Hemorrhagic activity

Hemorrhage was determined in groups of four mice (18–21 g), 2 hr after i.d. venom injection (1–16 μg) in 100 μl of PBS, by measuring the diameter of the hemorrhagic spot formed in the internal side of the skin (KONDO *et al.*, 1960). The skin was shaved before injection to visually verify the i.d. application of venom.

Myotoxic activity

Myonecrosis induced by the venom was evaluated by three criteria: histological observation, quantitation of plasma creatine kinase (CK; EC 2.7.3.2) activity, and by an MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide)-reduction assay. Groups of five mice received an i.m. venom injection (10–80 μg) in 100 μl of PBS in the right gastrocnemius. Control animals received a similar injection only of PBS. After 3 hr, a blood sample was obtained from the tail for the measurement of plasma CK activity, using a commercial colorimetric assay (Sigma No. 520) (GUTIÉRREZ *et al.*, 1984). After 24 hr, both gastrocnemius muscles were removed, weighed, and homogenized in PBS-1% Triton X-100. The MTT-reducing activity of each homogenate supernatant was determined by incubating 1 ml of homogenate with 0.2 ml of MTT (2.5 mg/ml PBS) for 90 min at 37°C, and then reading the absorbance at 570 nm against a 690 nm reference, as described in detail by LOMONTE *et al.* (1993*b*). The basis of this test is that residual MTT-reducing activity of the injected muscle correlates with the proportion

of unaffected cells in the tissue. For histological evaluation, muscle samples taken 24 after venom injection were fixed with Duboscq-Brasil solution (10% formalin, 50% ethanol, 6.5% acetic acid, 0.45% picric acid), embedded in paraffin and stained with a modified Masson trichrome method (ARCE, 1986).

Enzymatic activities

Proteolytic activity was tested by incubation of venom (30–500 μg/ml) with 1% casein in PBS for 30 min at 37°C. Proteins were then precipitated by addition of trichloroacetic acid, and the increase of absorbance at 280 nm of the supernatants was determined (LOMONTE and GUTIÉRREZ, 1983). Phospholipase A_2 activity was estimated by an indirect hemolytic radial diffusion assay in the presence of egg yolk phospholipids (GUTIÉRREZ *et al.*, 1988), using venom amounts ranging from 0.1–10 μg/well. Fibrinolytic activity was determined on equine fibrin clot using the radial diffusion assay described by GENÉ *et al.* (1989), with venom amounts ranging from 6–100 μg/well. Coagulant activity was tested on human plasma at 37°C, by adding varying amounts of venom (GENÉ *et al.*, 1989). Anticoagulant activity was determined according to the method of ALVARADO and GUTIÉRREZ (1988), by preincubating venom with platelet-poor human plasma at 37°C for 10 min, and then adding $CaCl_2$.

Antiserum production and cross-reactivity studies

A rabbit antiserum against *V. berus* venom was prepared to study the antigenic cross-reactivity between this venom and those from several crotalid species. Venom (1 mg) was emulsified with Freund's complete adjuvant and injected by the i.m. route. On days 21, 36, and 51, respectively, 1 mg doses were given using sodium alginate as a vehicle. The rabbit was bled 14 days after the last injection, and the antibody titer against *V. berus* venom was determined by enzyme-immunoassay (LOMONTE *et al.*, 1991). Serial dilutions of the immune serum were added to plastic wells coated with 1 μg of *V. berus* venom, and bound antibodies were detected using an anti-rabbit IgG-alkaline phosphatase conjugate and *p*-nitrophenol phosphate as substrate (Sigma). Normal rabbit serum at the same dilutions was used as a control. In order to evaluate the cross-reactivity between venoms from Old and New World vipers, an appropriate dilution of the anti-*V. berus* serum, corresponding to the linear region of response of the enzyme immunoassay, was tested against a collection of crotalid venoms from Costa Rica. All venoms were used at a concentration of 1 μg/well and binding of the rabbit antibodies was detected as described above. Cross-reactivity was expressed as a percentage, taking as 100% the absorbance readings obtained with *V. berus* venom as antigen.

Electrophoretic analyses and immunoblotting

Venom was analyzed by polyacrylamide (15%) gel electrophoresis in the presence of sodium dodecylsulphate (SDS-PAGE; LAEMMLI, 1970). Venom was separated at 200 V either unreduced or after reduction with 2-mercaptoethanol for 4 min at 95°C, in a Mini-Protean cell (Bio-Rad, Richmond, CA, U.S.A.). Proteins were stained with Coomassie blue R-250 and the gels were scanned on a GS300 Densitometer (Hoefer Instruments, San Francisco, CA, U.S.A.). Scans were analyzed with the GS365W Electrophoresis Data System (Hoefer) to determine the mol. wt and relative concentrations of the components. Reactivity of the rabbit anti-*V. berus* serum towards the different venom components was tested by immunoblotting. Unreduced venom was separated by SDS-PAGE and transferred to 0.45 μm nitrocellulose at 150 mAmp for 1.5 hr in a Mini-Transblot cell (Bio-Rad), using the buffer described by TOWBIN *et al.* (1979). Proteins on the nitrocellulose were reversibly-stained with amidoblack (SYU and KAHAN, 1987) to check for transfer efficiency. Strips were cut, blocked with bovine serum albumin and casein, and then incubated with either immune or normal rabbit sera. Bound antibodies were detected with an alkaline phosphatase anti-rabbit IgG conjugate, using nitroblue tetrazolium and 5-bromo-4-Cl-3-indolyl phosphate (Sigma) as substrates.

RESULTS

Lethal activity and local tissue-damaging effects

The estimated i.p. LD_{50} of *V. berus* venom was 0.86 ± 0.15 μg/g (14.6 ± 2.6 μg/mouse). The venom induced a marked and rapid edema in the mouse footpad (Fig. 1A), which peaked 0.5–1 hr after injection. This edema was dose-dependent 6–48 hr after injection, but not in the early stages (0.5–1 hr) of the response (Fig. 1A). *Vipera berus* venom also had strong hemorrhagic action, with a minimum hemorrhagic dose (amount of venom inducing a hemorrhage of 10 mm diameter) of 3.2 μg (Fig. 1B). Histological observations confirmed the conspicuous extravasation induced by this venom after i.m. injection (Fig.

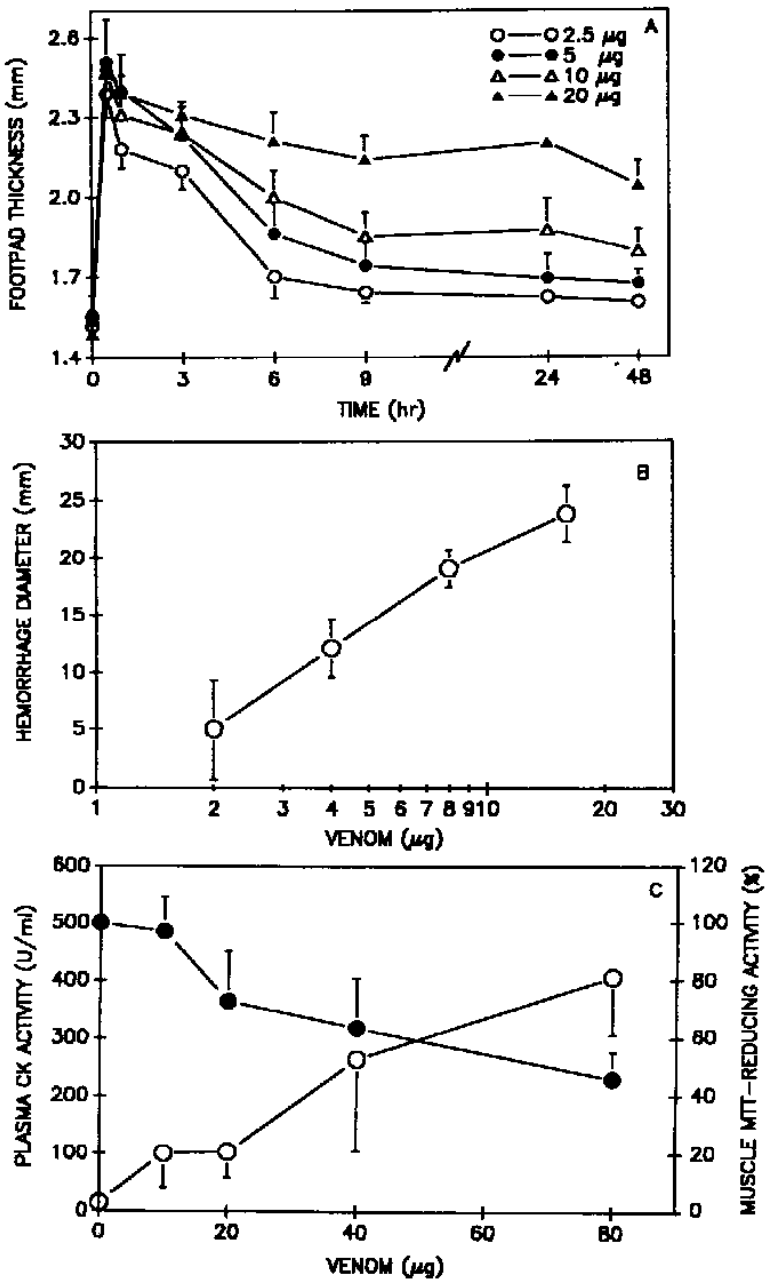

FIG. 1. *In vivo* LOCAL TISSUE-DAMAGING EFFECTS INDUCED IN THE MOUSE BY THE VENOM OF *Vipera berus*.

A. Edema-inducing activity of the venom in the mouse footpad. The indicated doses of venom were injected s.c. and local edema was estimated by measuring the changes in footpad thickness. Each point represents mean ± S.D. of five mice. B. Hemorrhagic activity of the venom in the mouse skin. Different venom doses were injected i.d. and the diameter of the hemorrhagic area was determined 2 hr later. Each point represents mean ± S.D. of four mice. C. Myotoxic activity of the venom in the mouse gastrocnemius muscle. Different venom doses were injected i.m. into the gastrocnemius and 3 hr later plasma creatine kinase levels (○) were determined. After 24 hr, the residual MTT-reducing activity (●) of the tissue was determined and expressed as a percentage of that of the normal contralateral muscle. Each point represents mean ± S.D. of five mice.

2A). In addition, this venom had myotoxic activity, as judged by biochemical indicators of skeletal muscle damage such as plasma CK levels or residual MTT-reducing activity of the injected tissue (Fig. 1C), and by histological evaluation (Fig. 2B).

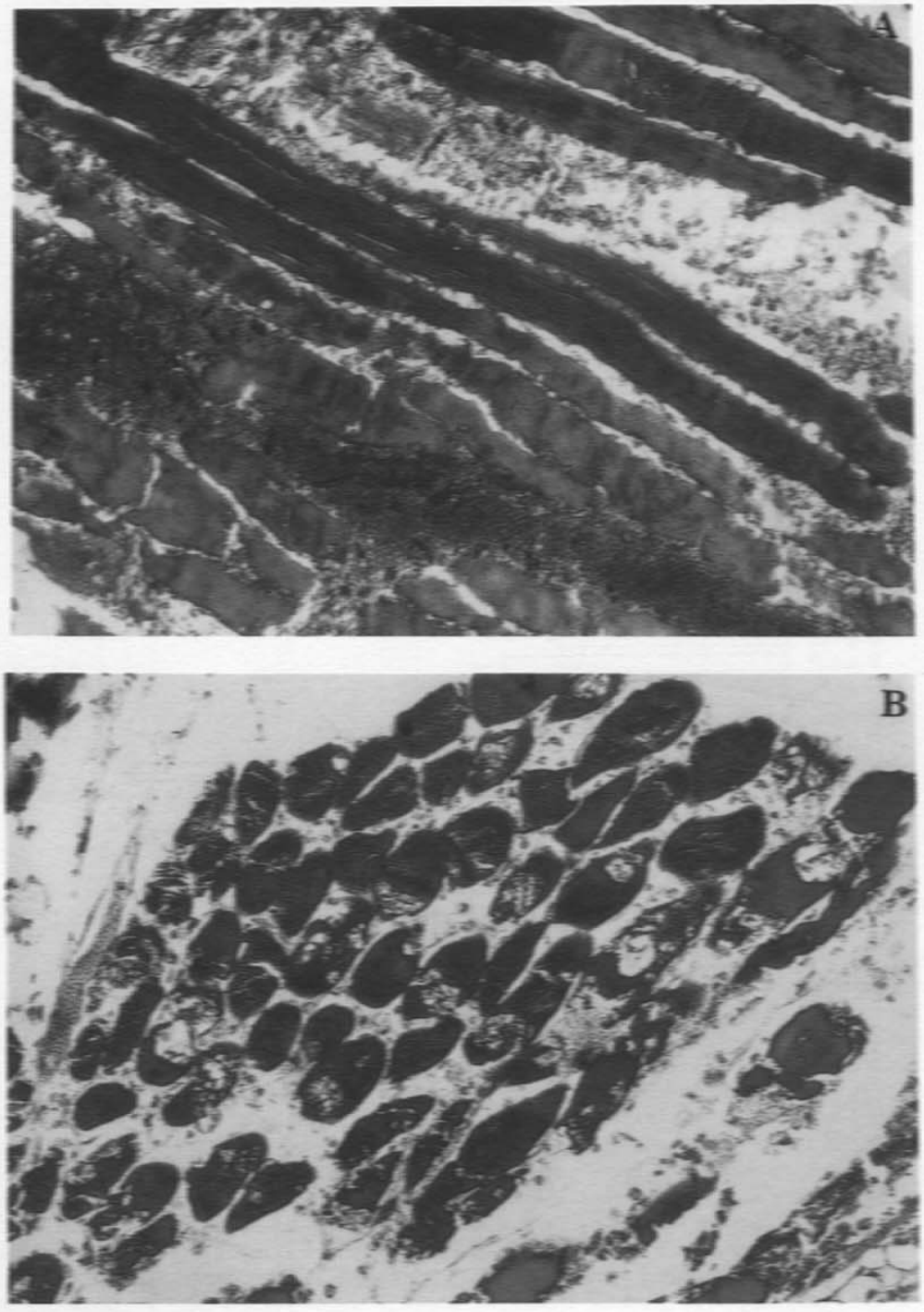

FIG. 2. HISTOLOGICAL ALTERATIONS INDUCED IN THE MOUSE GASTROCNEMIUS MUSCLE BY THE VENOM OF *Vipera berus*.

A. Venom (40 μg) was injected in the gastrocnemius muscle and the tissue was obtained after 24 hr. An area with widespread hemorrhage and a scarce inflammatory infiltrate is seen among the muscle fibers. Modified Masson trichrome stain. Magnification ×40. B. Same as above, showing an area of widespread myonecrosis. Magnification ×100.

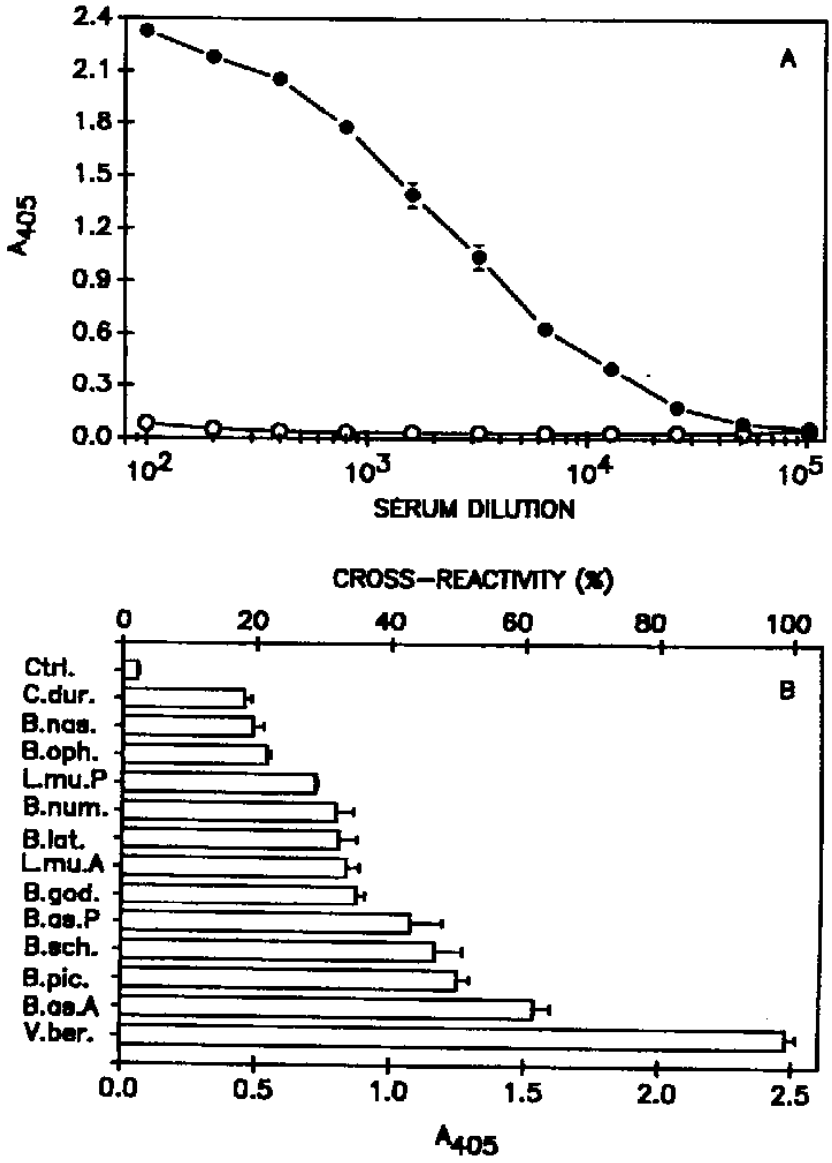

FIG. 3. TITRATION OF AN ANTI-*Vipera berus* RABBIT SERUM AND CROSS-REACTIVITY ANALYSIS USING VENOMS FROM CROTALID SPECIES, BY ENZYME-IMMUNOASSAY.

A. Titration curve of the anti-*V. berus* serum produced in rabbit. Serial dilutions of antiserum (●), or normal rabbit serum (○), were added to *V. berus*-coated plastic wells. Bound antibodies were detected using an anti-rabbit IgG-alkaline phosphatase conjugate and *p*-nitrophenol phosphate as substrate. Absorbances were determined at 405 nm (A_{405}). Each point represents the mean ± S.D. of triplicate wells. B. Cross-reactivity of the anti-*V. berus* antibodies against venoms of crotalid snake species from Costa Rica. *Vipera berus* specific antiserum was utilized at 1 : 1000 dilution and all venoms at 1 μg/well. Cross-reactivity is presented as a percentage of the absorbance readings obtained with the homologous venom (upper axis), or as absolute absorbance units (lower axis). Each bar represents the mean ± S.D. of triplicate wells. All venoms were also tested against normal rabbit serum as a control, giving readings (not shown) below the background level indicated in the figure (Ctrl, control of uncoated wells). C.dur, *Crotalus durissus durissus*; B.nas, *Bothrops nasutus*; B.oph, *B. ophryomegas*; L.mu. P, *Lachesis muta* (Pacific type); B.num, *B. nummifer*; B.lat, *B. lateralis*; L.mu.A, *L. muta* (Atlantic type); B.god, *B. godmani*; B.as.P, *B. asper* (Pacific type); B.sch, *B. schlegelii*; B.pic, *B. picadoi*; B.as.P, *B. asper* (Pacific type); V.ber, *V. berus*.

Enzymatic activities

Vipera berus venom had moderate proteolytic activity on casein, with a specific activity of 59.2 units/mg in the assay system utilized. Phospholipase A_2 activity was detected with

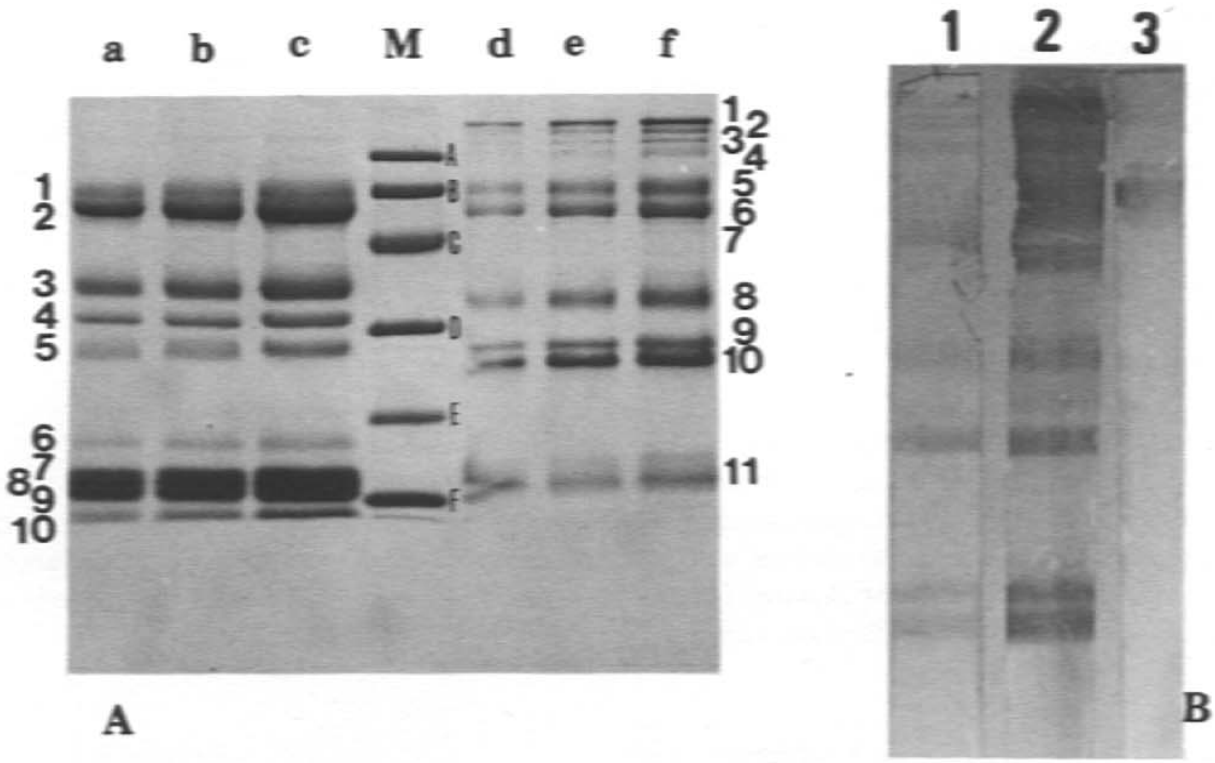

FIG. 4. ELECTROPHORETIC AND IMMUNOBLOTTING ANALYSES OF *Vipera berus* VENOM.
A. SDS-polyacrylamide gel (15%) electrophoresis of the venom after reduction with 2-mercaptoethanol (lanes a,b,c, containing 20, 30, and 50 μg, respectively), or unreduced (lanes d,e,f, containing 20, 30, and 50 μg, respectively). Coomassie blue R-250 stain. M = mol. wt markers (A. 94,000; B. 67,000; C. 43,000; D. 30,000; E. 20,100; F. 14,400). Band numbers, starting from the top, correspond to the desitometric data presented in Table 1. B. Immunoblotting of *V. berus* venom antigens (unreduced) using the rabbit antiserum. 1. Protein pattern revealed by mild (reversible) amidoblack stain; 2. reactivity of the anti-*V. berus* rabbit serum, 1:25 dilution; 3. normal rabbit serum control, 1:25 dilution.

TABLE 1. QUANTITATIVE DENSITOMETRIC ANALYSIS OF THE COMPONENTS OF *Vipera berus* VENOM SEPARATED BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS*

Unreduced venom			Reduced venom		
Band	Mol. wt	%	Band	Mol. wt	%
1	125,800	8.2	1	72,600	9.9
2	114,900	3.6	2	62,000	19.1
3	106,100	2.0	3	37,000	13.0
4	98,500	2.8	4	31.400	5.8
5	68,000	13.1	5	26,300	5.6
6	57,200	13.4	6	18,000	4.9
7	54,000	1.9	7	16,000	7.7
8	32,800	19.5	8	15,600	11.7
9	25,700	7.7	9	15,100	15.6
10	23,800	18.9	10	14,200	6.7
11	15,200	9.0			

*Data corresponding to the electrophoretic separations shown in Fig. 4A.

0.3 μg venom/well, or more, by the indirect hemolytic assay, and the minimum hemolytic dose (venom dose causing a lytic halo of 15 mm diameter) was approximately 1.5 μg. The fibrinolytic activity was low, since even with 100 μg venom/well, a lytic halo of 10 mm diameter in the fibrin clot (minimum fibrinolytic dose) was not achieved. This venom did not show coagulant activity on human plasma *in vitro*, even when as much as 200 μg venom was tested. However, a moderate, but significant anticoagulant activity *in vitro* was observed. Using 20 mg of venom, the plasma recalcification time was 19.8 ± 6.8 min, in contrast to 7.0 ± 0.4 min for control. At a dose of 200 μg of venom, plasma was unclottable during a 2 hr observation period.

Cross-reactivity with crotalid venoms

The titer of anti-*V. berus* antibodies detected in the serum from a hyperimmunized rabbit using the enzyme-immunoassay was approximately 1 : 40,000 (Fig. 3A). Using this serum, significant cross-reactivities with several crotalid venoms were obtained, ranging from 62% for the venom of *Bothrops asper* (Atlantic region of Costa Rica) to 18%, in the case of *Crotalus durissus durissus* venom (Fig. 3B).

Electrophoretic and immunoblotting analyses

The SDS-PAGE patterns of *V. berus* venom (unreduced and reduced) are shown in Fig. 4A. The mol.wt and relative proportions of the components resolved are presented in Table 1. Immunoblots indicated that all venoms bands resolved by SDS-PAGE and visible by protein stain were recognized by antibodies present in the rabbit antiserum (Fig. 4B). There was an unexpected reaction of normal rabbit serum antibodies with the venom band of 68,000 mol. wt (Fig. 4B). The experiment was repeated using normal rabbit sera from two different animals, with the same result.

DISCUSSION

The venom of *V. berus* was highly lethal to mice, with an LD_{50} (i.p.) of 0.86 μg/g body weight. This estimation is very similar to that report by RUSSELL (1967) and by MINTON (reviewed by TU, 1977) of 0.80 μg/g. This lethal potency, similar to that of most *Vipera* venoms (reviewed by TU, 1977), was higher than that of all Central American crotalid venoms, with the exception of *Crotalus durissus durissus* (BOLAÑOS, 1972). The lethal potency of *V. berus* venom was comparable to that of neurotoxic Elapidae venoms, such as that of *Micrurus* spp. (BOLAÑOS, 1972). However, these estimations cannot be extrapolated to the human, in which a relatively low fatality rate is observed (HABERMEHL, 1981). Nevertheless, severe cases of envenomation due to *V. berus* may occur (HABERMEHL, 1981; STAHEL *et al.*, 1985; CEDERHOLM and LENNMARKEN, 1987; GONZALEZ, 1991).

Experimentally, *V. berus* venom induced significant local tissue-damaging effects, including edema, hemorrhage, and myonecrosis. The local edema formation was characterized by rapid onset, reaching a maximum of 0.5–1 hr. After this early peak, the persistence of edema was related to venom dose. With the higher doses tested, considerable swelling of the footpad persisted for more than 2 days. This edema pattern was very similar to that recently described for *Bothrops asper* venom (LOMONTE *et al.*, 1993*a*).

A potent hemorrhagic action of the venom was observed, both histologically and by the quantitative macroscopic skin test. The estimated potency (3.2 μg minimum hemorrhagic dose) was slightly lower than that of most crotalid venoms of the genus *Bothrops* studied (GUTIÉRREZ *et al.*, 1985). A hemorrhagic protein has been isolated from *V. berus* venom and biochemically characterized as a zinc-containing metalloprotease of 56,300 mol. wt, with proteolytic activity on casein and fibrinogen (SAMUEL and SIIGUR, 1990).

Myonecrosis has not previously been described as an effect of *V. berus* venom. These results showed a moderate, but clear myotoxic action of this venom at the site of injection. Skeletal muscle damage was corroborated histologically, as well as inferred by the biochemical tests. There was a dose-dependent increase of plasma CK activity, and decrease of muscle residual MTT-reducing activity, with good correlation between these two parameters. The increase in plasma CK activity obtained was approximately half that induced by the same dose of a known myotoxic venom such as *B. asper* (data not shown). Myotoxic activity in venoms from several *Vipera* species, although not in that of *V. berus* from Czechoslovakia, was recently reported by MEBS and LANGELUDECCKE (1992), using a qualitative histological technique. Geographical variations may account for the difference with the present study. The lack of reports on the myotoxic action of this venom may be due to the low amounts of venom injected following human snakebites; however, it could also have been missed in clinical evaluations.

The proteolytic activity of the venom, using casein as substrate, was 59.2 U/mg. This value is lower than that of most crotalid venoms, tested under the same conditions (LOMONTE and GUTIÉRREZ, 1983). Despite the fact that hemorrhagic factors found in snake venoms are proteases (BJARNASON and FOX, 1988–89), studies performed with several venoms indicate that hemorrhagic and proteolytic activities do not correlate (GUTIÉRREZ *et al.*, 1985), probably because hemorrhagic toxins are metalloproteases which act only on highly specific substrates (BJARNASON and FOX, 1988–89). Present results provide further support to this hypothesis, since *V. berus* venom was highly hemorrhagic but only slightly proteolytic. However, protease inhibitors have been found in this venom (SIIGUR *et al.*, 1988) in addition to proteases (SIIGUR *et al.*, 1979).

The phospholipase A_2 activity of *V. berus* venom has been described previously (BÜCHERL *et al.*, 1968; SIIGUR *et al.*, 1979; TAN and PONNUDURAI, 1990). BOFFA *et al.*, (1976) purified a phospholipase A_2 occurring as a 14,000 mol. wt monomer or 39,000 mol. wt trimer, with anticoagulant activity. Both phospholipase and anticoagulant activities were confirmed in this study of the whole venom. Very low fibrinolytic activity was observed, which agrees with the low proteolytic activity. The venom lacked the ability to directly activate coagulation of human plasma *in vitro*, even at high concentrations (700 μg/ml).

Immunoblotting using *V. berus* antiserum showed that all components resolved by SDS-gel electrophoresis were recognized by antibodies, and therefore immunogenic. Since viperids and crotalids probably evolved from common ancestors (PHELPS, 1981), some degree of antigenic conservation is to be expected. Indeed, using an enzyme-immunoassay, variable degrees of cross-reactivity were shown between antigens of *V. berus* and of crotalid venoms. Interestingly, the venoms with high cross-reactivity with *V. berus* venom in the enzyme-immunoassay, were those with high hemorrhage activities (*B. asper* Atlantic type, *B. picadoi*). MANDELBAUM *et al.* (1989) have reported cross-neutralization of *V. lebetina* venom hemorrhagic activity by antisera raised against purified hemorrhagic toxins from *B. jararaca* and *B. neuwiedi*. Recent studies by BORKOW *et al.* (1993) indicate antigenic cross-reactivity between purified hemorrhagic factors from *B. asper* and *Vipera*

palestinae. MEBS *et al.* (1988) have also documented considerable cross-neutralizations of hemorrhagic activities of venoms from crotalid and viperid snakes, using a variety of antivenoms. Thus, at least part of the cross-reactivity observed in this work is probably due to common antigenic structures on hemorrhagic toxins.

Acknowledgements—We thank JAVIER NÚÑEZ and MARJORIE ROMERO for expert help in the laboratory, and Drs GUSTAVO ROJAS and FERNANDO CHAVES for valuable discussions and advice. B.L. and J.M.G. were supported by the Costa Rican National Scientific and Technological Research Council (CONICIT) during this study. The contribution of the Ellen, Walter and Lennart Hesselman's Foundation is also gratefully acknowledged.

REFERENCES

ALVARADO J. and GUTIÉRREZ, J. M. (1988) Anticoagulant effect of myotoxic phospholipase A_2 isolated from the venom of the snake *Bothrops asper* (Viperidae) *Rev. Biol. Trop.* **36**, 563–565.

ARCE, V. (1986) A comparative histochemical study of bulbo-urethral and urethral glands in five rodent species, Ph.D. Thesis, University of Cambridge, U.K.

BJARNASON, J. B. and FOX, J. W. (1988–89) Hemorrhagic toxins from snake venoms. *J. Toxicol. Rev.* **7**, 121–209.

BOFFA, M. C., WINCHENNE, J. J., LUCIEN, N. and BOFFA, G. A. (1976) The anticoagulant phospholipase A_2 from *Vipera berus* venom. In: *Toxins: Animal, Plant and Microbial*, pp. 319–331 (ROSENBERG, P., Ed.). Oxford: Pergamon Press.

BOLAÑOS, R. (1972) Toxicity of Costa Rican snake venoms for the white mouse. *Am. J. trop. Med. Hyg.* **21**, 360–363.

BORKOW, G., GUTIÉRREZ, J. M. and OVADIA, M. (1993) Isolation and characterization of synergistic hemorrhagins from the venom of the snake *Bothrops asper*. *Toxicon* (in press).

BÜCHERL, W., BUCKLEY, E. and DEULOFEU, V. (1968) *Venomous Animals and their Venoms*, 707 pp. New York: Academic Press.

CEDERHOLM, I. and LENNMARKEN, C. (1987) *Vipera berus* bites in children. Experience of early antivenom treatment. *Acta Pediatr. Scand.* **76**, 682–684.

GENÉ, J. A., ROY, A., ROJAS, G., GUTIÉRREZ, J. M. and CERDAS, L. (1989) Comparative study on coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of Costa Rican Crotaline snake venoms and their neutralization by a polyvalent antivenom. *Toxicon* **27**, 841–848.

GONZALEZ, D. (1991) Snakebite problems in Europe. In: *Reptile Venoms and Toxins, Handbook of Natural Toxins*, Vol. 5, pp. 687–751 (TU, A. T., Ed.). New York: Marcel Dekker.

GUTIÉRREZ, J. M., OWNBY, C. L. and ODELL, G. V. (1984) Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon* **22**, 115–128.

GUTIÉRREZ, J. M., GENÉ, J. A., ROJAS, G. and CERDAS, L. (1985) Neutralization of proteolytic and hemorrhagic activities of Costa Rican snake venoms by a polyvalent antivenom. *Toxicon* **23**, 887–893.

GUTIÉRREZ, J. M., AVILA, C., ROJAS, E. and CERDAS, L. (1988) An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **26**, 411–413.

HABERMEHL, G. G. (1981) *Venomous Animals and their Toxins*, 195 pp. Berlin: Springer.

KONDO, H., KONDO, S., IKEZAWA, H., MURATA, R. and OHSAKA, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venon. *Jpn. J. Med. Sci. Biol.* **13**, 43–51.

LAEMMLI, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.

LOMONTE, B. and GUTIÉRREZ, J. M. (1983) La actividad proteolítica de los venenos de serpientes de Costa Rica sobre la caseína. *Rev. Biol. Trop.* **31**, 37–40.

LOMONTE, B., GUTIÉRREZ, J. M., ROJAS, G. and CALDERÓN, L. (1991) Quantitation by enzyme-immunoassay of antibodies against *Bothrops* myotoxins in four commercially-available antivenoms. *Toxicon* **29**, 695–702.

LOMONTE, B., TARKOWSKI, A. and HANSON, L. Å. (1993*a*) Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* (in press).

LOMONTE, B., GUITIÉRREZ, J. M., ROMERO, M., NÚÑEZ, J., TARKOWSKI, A. and HANSON, L. Å. (1993*b*) An MTT-based method for the *in vivo* quantification of myotoxic activity of snake venoms and its neutralization by antibodies. *J. Immun. Meth.* (in press).

MANDELBAUM, F. R., SERRANO, S. M. T., SAKURADA, J. K., RANGEL, H. A. and ASSAKURA, M. T. (1989) Immunological comparison of hemorrhagic principles present in venoms of the Crotalinae and Viperinae subfamilies. *Toxicon* **27**, 169–177.

MEBS, D., POHLMANN, S. and VON TENSPOLDE, W. (1988) Snake venom hemorrhagins: neutralization by commercial antivenoms. *Toxicon* **26**, 453–458.

MEHRTENS, J. M. (1987) *Living Snakes of the World*, 480 pp. New York: Sterling.

PHELPS, T. (1981) *Poisonous Snakes*, 237 pp. London: Blandford Press.

RUSSELL, F. E. (1967) Pharmacology of animal venoms. *Clin. pharmac. Ther.* **8,** 849–873.

SAMEL, M. and SIIGUR, J. (1990) Isolation and characterization of hemorrhagic metalloproteinase from *Vipera berus berus* (common viper) venom. *Comp. Biochem. Physiol.* **97,** 209–214.

SIIGUR, E., SIIGUR, J. NOMMEOTS, M. and ILOMETS, T. (1979) Fractionation and enzymatic activities of common viper (*Vipera berus berus*) venom. *Toxicon* **17,** 623–630.

SIIGUR, J., ARUMAE, U., NEUMAN, T., SAMEL, M., SIIGUR, E. and SAARMA, M. (1986) Isolation and characterization of nerve growth factor from *Vipera berus berus* (common viper) venom. *Comp. Biochem. Physiol.* **83B,** 621–625.

SIIGUR, E., SAMEL, M. and SIIGUR, I. (1988) Ingibitory tripsina i khimotripsina iz iada gadiuki. *Biokhimiia* **53,** 302–308.

STAHEL, E., WELLAUER, R. and FREYVOGEL, T. A. (1985) Vergiftungen durch einhei mische Vipern (*Vipera berus* und *Vipera aspis*). Eine retrospektive studie an 113 patienten. *Schweiz Med. Wochenschr.* **115,** 890–896.

SYU, W. J. and KAHAN, L. (1987) Use of protein-stained immunoblots for unequivocal identification of antibody specificities. *J. Immun. Meth.* **103,** 247–252.

TAN, N. and PONNUDURAI, G. (1990) A comparative study of the biological properties of venoms from snakes of the genus *Vipera* (true adders). *Comp. Biochem. Physiol.* **96B,** 683–688.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **76,** 4350.

TREVORS, J. T. (1986) A BASIC program for estimating LD50 values using the IBM-PC. *Bull. environ. Contam. Toxicol.* **37,** 18–26.

TU, A. T. (1977) *Venoms: Chemistry and Molecular Biology,* 560 pp. New York: Wiley.

VAN LOVEREN, H., KATO, K., RATZLAFF, R. E., MEADE, R., PTAK, W. and ASKENASE, P. W. (1984) Use of micrometers and calipers to measure various components of delayed-type hypersensitivity ear swelling reactions in mice. *J. Immun. Meth.* **67,** 311–319.

III

Journal of Immunological Methods, 161 (1993) 231–237
Elsevier Science Publishers B.V.

JIM 06687

An MTT-based method for the in vivo quantification of myotoxic activity of snake venoms and its neutralization by antibodies

Bruno Lomonte [a,b], José María Gutiérrez [a], Marjorie Romero [a], Javier Núñez [a], Andrej Tarkowski [b] and Lars Å. Hanson [b]

[a] *Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica and* [b] *Department of Clinical Immunology, University of Göteborg, Göteborg, Sweden*

(Received 19 November 1992, revised received 12 January 1993; accepted 18 January 1993)

The reduction of the tetrazolium compound MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was used as the basis for the development of a simple method for the quantitative estimation of metabolically active skeletal muscle tissue remaining after in vivo venom-induced myonecrosis. Using the venom of the snake *Micrurus nigrocinctus* as a potent myotoxic agent, this MTT-based technique was evaluated in comparison with available methods based on the measurement of creatine kinase (CK) activity, and a quantitative histological technique considered as a reference. Homogenates of the gastrocnemius muscle prepared in the presence of 1% Triton X-100 reduced MTT and this activity correlated closely with the number of viable cells in the tissue, as determined by histological evaluation. After venom injection, residual MTT-reducing activity of muscle homogenates showed higher correlation to the myonecrosis index obtained by histological analysis, than residual muscle CK activity. Using the new MTT-based assay, the ability of an anti-*M. nigrocinctus* equine antivenom to neutralize venom myotoxins was studied. The myotoxic activity of the venom was completely neutralized using 4 ml antivenom/mg venom, with a 50% effective dose (ED_{50}) value of about 2.5 ml/mg. The MTT-based method described should be useful in the estimation and standardization of anti-myotoxic potency of antivenoms, and in the screening of other neutralizing agents, as a convenient and reliable alternative to the time-consuming quantitative histological methods.

Key words: MTT; Myonecrosis; Antibody neutralization; Snake venom; *Micrurus*

Introduction

Heterologous immunoglobulin preparations with antivenom activity have been used for the treatment of snake bites since the pioneer work of Calmette in 1894 (reviewed by Grabar, 1986). However, only in recent years has it become clear that, in addition to being effective in the neutralization of lethal components of venoms, antibody preparations should also be standardized for neutralization of other clinically relevant venom effects (W.H.O., 1981). One of these effects is myonecrosis, a common consequence of envenomations by many snake species, which can be responsible for permanent sequelae (Mebs and Ownby, 1990; Harris and Cullen, 1990). One limi-

Correspondence to: B. Lomonte, Department of Clinical Immunology, University of Göteborg, Guldhedsgatan 10, S-41346, Göteborg, Sweden. Tel.: 46-31-604692; Fax: 46-31-826791.

tation in the assessment of the anti-myotoxic potency of antivenoms in animal models has been the lack of simple and reliable methods for the quantitative estimation of myonecrosis, as an alternative to the time-consuming quantitative histological methods. In recent years, a frequently used assay has been based on the quantification of plasma creatine kinase (CK) activity, due to the release of this enzyme from damaged skeletal muscle fibres (Gutiérrez et al., 1980; Nakada et al., 1980; 1984; Mebs et al., 1983). Other enzymes have also been evaluated as indicators of myonecrosis (Preston et al., 1990). One limitation of this approach might be the influence of pharmacokinetic parameters, such as plasma half-life and body distribution of the enzyme, as well as optimal sampling time. Another potential pitfall is that enzymes could escape from the cytosol of reversibly damaged cells which afterwards regain their normal condition (Suarez-Kurtz, 1982).

In the present work, the reduction of the tetrazolium compound MTT, a commonly used reaction for the assessment of cell viability in vitro (Mosmann, 1983; Denizot and Lang, 1986; Hansen et al., 1989), was used for the quantitative estimation of metabolically active skeletal muscle tissue remaining after in vivo venom-induced myonecrosis. A technique was developed and evaluated in comparison with existing methods based on the measurement of CK activity, and a quantitative histological technique considered as a reference. The venom of the Central American coral snake *Micrurus nigrocinctus* was used as a potent myotoxic agent (Gutiérrez et al., 1986). The usefulness of the new MTT-based method in the evaluation of the specific myotoxic-neutralizing ability of a commercially-available antivenom was investigated.

Materials and methods

Snake venom and antiserum

The venom of *Micrurus nigrocinctus* was a pool obtained from 50–60 specimens collected in Costa Rica and kept at the Instituto Clodomiro Picado. It was lyophilized and stored at −20°C until used. The equine anti-*M. nigrocinctus* serum (ammonium sulphate globulin fraction, batch 207A89LF) was produced at the Instituto Clodomiro Picado as described by Bolaños and Cerdas (1980).

Dose-response analysis for myotoxic activity

Swiss Webster mice (16–18 g body weight) received a sublethal i.m. venom injection into the right gastrocnemius, in a volume of 50 μl of 0.12 M NaCl, 40 mM sodium phosphate buffer, pH 7.2 (PBS). Venom doses ranged from 0.6 to 20 μg/mouse. After 24 h the animals were killed by ether inhalation and both the injected and the contralateral gastrocnemius muscles were dissected out. Each muscle was weighed and immediately homogenized in 4 ml of PBS containing 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA), using a Brinkmann PT10/35 homogenizer (Polytron, Switzerland). After centrifugation at 1000 × g for 10 min, each supernatant was assayed for either MTT-reducing or CK activities, as described below. Each group comprised 5–6 mice. The time used for the development of myonecrosis (24 h) was selected after a time-course experiment over 1–24 h, using a fixed venom dose of 20 μg/mouse.

Quantification of MTT-reducing activity

1 ml of each muscle homogenate supernatant was mixed with 0.2 ml of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium ·bromide) solution (2.5 mg/ml PBS) in a 1.5 ml microfuge vial and incubated in the dark at 37°C for 90 min. Final absorbances were determined at 570 nm against a 690 nm reference (Denizot and Lang, 1986) on a Shimadzu UV-160 spectrophotometer. Each absorbance value was divided by the corresponding muscle weight, in order to compensate for minor variations during dissection of the tissue. The specific activity of the injected muscle was expressed as a percentage of the control (contralateral) muscle, representing the residual MTT-reducing activity after myonecrosis.

Quantification of creatine kinase (CK) activity

The supernatant of each muscle homogenate was diluted 1/40 with distilled water and 7 μl of this dilution were assayed for CK (EC 2.7.3.2) activity using a colorimetric assay (Sigma no. 520). Activity was expressed as U/ml, 1 U being de-

fined as the phosphorylation of 1 nmol of creatine per min at 25°C. The activity of each muscle supernatant was divided by its weight, and the specific activity of the injected muscle was expressed as a percentage of the corresponding contralateral control muscle, representing the residual CK activity after myonecrosis.

Since plasma CK activity has been frequently used as a quantitative estimator of venom-induced myonecrosis, a similar dose-response experiment was performed in which plasma CK was quantified for comparison with residual MTT-reducing or CK activities in muscle. Groups of five mice were bled from the tip of the tail into heparinized capillary tubes 3 h after venom injection, and then plasma CK activity was quantified as described. This sampling time was previously determined to correspond to the peak of the CK activity (Gutiérrez et al., 1980).

Histological analysis

Venom (0.6–20 μg) was injected by the i.m. route into the right gastrocnemius of six groups of four mice, in 50 μl of PBS. Control mice received a 50 μl PBS injection under otherwise identical conditions. After 24 h, animals were killed and the injected gastrocnemius muscle was removed. The muscle was cut transversally in the middle portion and immediately immersed in Duboscq-Brasil fixative (10% formalin, 50% ethanol, 6.5% acetic acid, 0.45% picric acid). Tissue samples were then dehydrated in ethanol and embedded in paraffin. The orientation of the tissue was carefully controlled during embedding in order to obtain cross-sections. Transverse sections (5–8 μm) were stained with a modified Masson trichrome stain (Arce, 1986). Video recordings encompassing the whole area of each section were made, and the total number of necrotic and normal muscle cells were counted for each sample. A 'Necrosis Index' was calculated as the number of necrotic cells/total number of cells.

Neutralization of myotoxicity by antivenom

A fixed amount of venom was mixed with various amounts of equine antivenom globulin in order to obtain different antivenom/venom ratios, ranging from 0 to 8 ml antivenom/mg venom. The mixtures were incubated at 37°C for 30 min and then injected by the i.m. route into the right gastrocnemius of six groups of six mice, in a total volume of 100 μl, containing 15 μg of venom (Gutiérrez et al., 1991). Control groups received either venom alone or PBS. After 24 h, residual MTT-reducing activity of the injected muscles was determined as described.

Results

Selection of the MTT assay conditions

Incubation of gastrocnemius homogenates with MTT at 37°C resulted in the formation of formazan in a time-dependent manner. Using an incubation time of 90 min, the final absorbance values ranged from 0.2 to 1.7. The purple product remained soluble in the PBS-Triton buffer, and this was always confirmed by centrifugation and subsequent visual inspection of reaction vials. An experiment designed to optimise the tissue sampling time showed a rapid decrease in the residual MTT-reducing activity after venom injection, followed by a levelling out of such activity (Fig. 1). Thus, the sampling time chosen was 24 h, when the decrease in residual MTT-reducing activity was maximal (Fig. 1).

Evaluation of the dose-response curves

The quantitative estimation of myonecrosis induced by different venom doses, using either his-

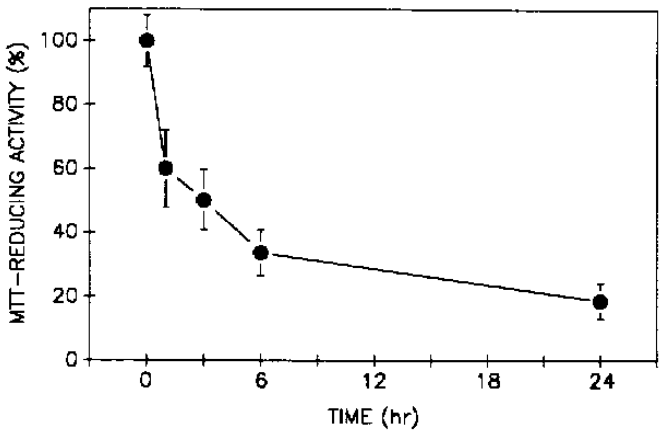

Fig. 1. Time-course of gastrocnemius MTT-reducing activity after the injection of 20 μg of *Micrurus nigrocinctus* venom. Groups of mice received an i.m. injection of venom in the gastrocnemius, and at the time points indicated the residual MTT-reducing activity of the muscle was determined. Each point represents the mean ± SD of 5–6 mice.

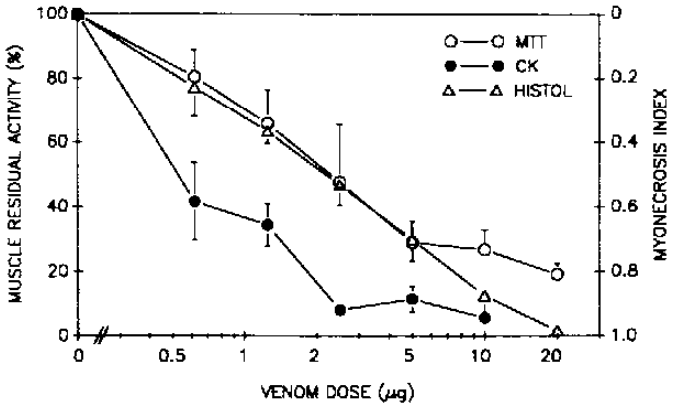

Fig. 2. Dose-response analysis of the myonecrosis induced by the venom of *Micrurus nigrocinctus*. Groups of mice received an i.m. injection of venom in the gastrocnemius, and 24 h later, myonecrosis was quantified by histological analysis (△). In parallel, residual CK (●) and MTT-reducing (○) activities were determined in the injected muscle. Each point represents the mean ± SD of 5–6 mice, except for quantitative histology, in which four mice per group were utilized.

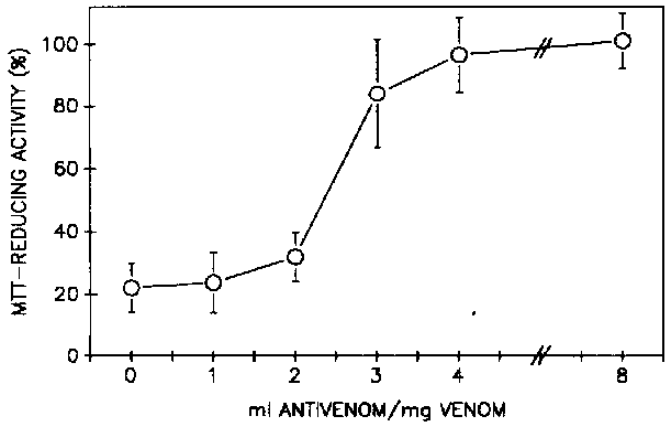

Fig. 4. Neutralization of venom myotoxic activity by an equine antivenom to *Micrurus nigrocinctus*. Mixtures of venom and antivenom at the ratios indicated were incubated at 37°C for 30 min and then injected i.m. into the gastrocnemius of groups of six mice. The venom challenge dose was 15 μg. After 24 h, the residual MTT-reducing activity of the injected muscle was determined. Each point represents mean ± SD.

tological analysis, residual MTT-reducing activity, or residual CK activity, is compared in Fig. 2. The quantitative histological analysis showed a better agreement with the residual MTT-reducing activity than with the residual CK activity (Fig. 2). Residual CK values overestimated myonecrosis compared with MTT-reducing activity, since at doses of 2.5 μg and higher, almost all the CK activity in the tissue was lost, despite the presence of a significant proportion of normal fibres (Figs. 2 and 5). In contrast, MTT-reducing activity decreased to a very similar extent as the necrotic index. At high venom doses, at which histologic evaluation indicated a necrotic index of almost 1.0, the dose-response curve for residual MTT-reducing activity tended to reach a plateau around 20% (Fig. 2). On the other hand, quantification of plasma CK levels showed a continuous increase over the entire venom dose range tested (Fig. 3). This increase was maintained even at doses at which the injected gastrocnemius was almost completely depleted of CK activity (Fig. 2).

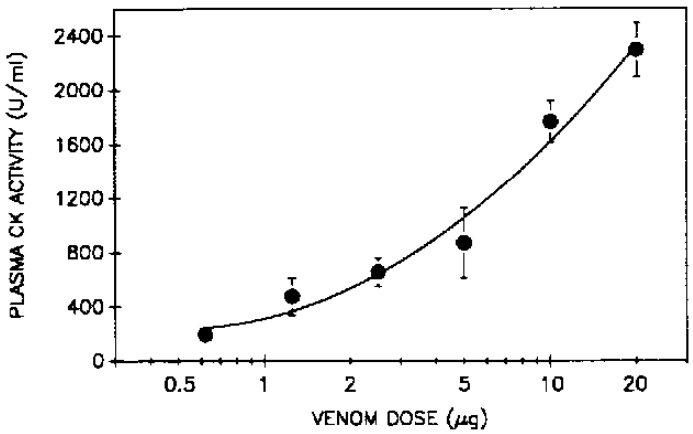

Fig. 3. Dose-response curve of the plasma creatine kinase increase induced by the venom of *Micrurus nigrocinctus*. Groups of five mice received an i.m. injection of venom in the gastrocnemius, and 3 h later plasma CK levels were determined. Each point represents the mean ± SD.

Use of the MTT-based method for neutralization assays

Using a venom challenge dose of 15 μg, the ability of an anti-*M. nigrocinctus* equine antivenom to neutralize myotoxicity was tested by in vitro preincubation and subsequent i.m. administration, using the MTT-based method (Fig. 4). The neutralization curve showed a complete inhibition of venom myotoxins at a ratio of antivenom/venom of 4 ml/mg or higher, on the basis of the residual MTT-reducing activity of the injected muscle. The 50% effective dose (ED_{50}), i.e., the amount of antivenom neutralizing 50% of

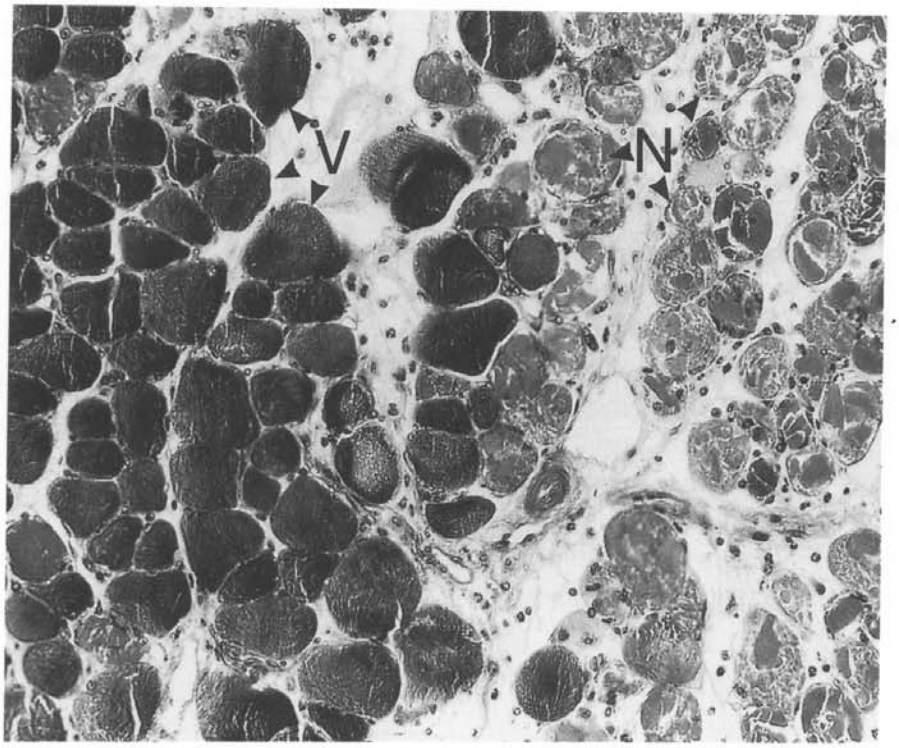

Fig. 5. Photomicrograph of a section of mouse gastrocnemius 24 hr after injection of 2.5 μg of *Micrurus nigrocinctus* venom. Notice the presence of viable muscle fibres (V) and necrotic fibres (N). A mild inflammatory infiltrate is present.

the myotoxic effect of venom, was approximately 2.5 ml antivenom/mg venom.

Discussion

Although quantitative histological methods are the most accurate and reliable in measuring the extent of skeletal muscle damage (Ownby et al., 1982; Kouyoumdjian et al., 1986; Preston et al., 1990; McLoon et al., 1991), they require a considerable amount of work and equipment. Therefore, the study of myotoxic factors in snake venoms and their neutralization by antibodies has been limited, in many instances, to qualitative analyses. Several alternative methods have been devised for the quantitative estimation of myonecrosis. The most utilized have been those based on CK determination, either in plasma or in muscle. A potential problem with the use of CK as an estimator of myonecrosis is the fact that this enzyme could be released from muscle cells due to conditions that do not necessarily lead to irreversible damage and cell death (Ownby et al., 1982; Suarez-Kurtz, 1982). Thus, there is a need to develop techniques for measuring myonecrosis and its neutralization. In the present work, we assessed the use of an MTT reduction assay for this purpose.

The results obtained showed that muscle homogenates prepared in the presence of 1% Triton X-100 had detectable MTT-reducing activity which correlated closely with the number of viable cells in the tissue as determined by quantitative histological evaluation. Residual MTT-reducing activity of the muscle homogenates correlated better than residual CK activity, with the myonecrosis index obtained by histological analysis. The discrepancy between the residual CK and MTT-reducing activities may have been due to the fact that, although both parameters are measuring muscle cell damage, they reflect different aspects of cell perturbation. Reduction of MTT depends on active mitochondrial metabolism, i.e., generation of reductive radicals in energetic metabolism (Mosmann, 1983), whereas CK release from muscle is due to an increase in the permeability of plasma membrane to this enzyme (Suarez-Kurtz, 1982). It is likely that changes in muscle CK content reflect more subtle cell alterations than changes in the ability of muscle to reduce MTT, since CK release may occur in cells

with normal mitochondrial function but altered sarcolemmal permeability. Indeed, it has been demonstrated that viable muscle cells can release CK in reversible stages of cell injury (Ownby et al., 1982) and as a consequence of osmotic changes (Suarez-Kurtz, 1982). In contrast, studies on ischemic muscle have shown that there is a correlation between the lack of nitroblue tetrazolium reduction and irreversible cell injury (Labbe et al., 1988).

Although the residual MTT-reducing activity values followed closely the myonecrosis values obtained histologically, a baseline of about 20% activity was observed at dose levels where muscle necrosis was nearly complete. A possible explanation for this 'background' activity may have been the contribution to the MTT reduction of myotoxin resistant cell types in the tissue other than skeletal muscle fibres. Nevertheless, despite this basal level of activity there is an adequate working range in the dose-response curve (between 100% and 20%) for an accurate estimation of myonecrosis and its neutralization.

An interesting observation regarding the use of plasma CK determination for the assessment of myonecrosis was the finding that at venom doses of 10 and 20 μg, where there was an almost complete necrosis and depletion of CK from the injected gastrocnemius muscle, plasma CK levels continued to rise. This implies that at high venom doses, CK release occurs not only at the site of injection, but also at adjacent or distant sites. Thus, plasma CK levels probably reflect both local and systemic myotoxicity. This would represent an advantageous use of plasma CK in experimental models where systemic muscle damage is to be evaluated.

Using the new MTT-based assay, the ability of an anti-*M. nigrocinctus* therapeutic antivenom to neutralize venom myotoxins was tested. Results confirmed that the antivenom contains neutralizing antibodies against myotoxic factors present in this venom. Myotoxic activity was completely neutralized at the ratio of 4 ml antivenom/mg venom. The ED_{50} obtained from the neutralization curve (2.5 ml antivenom/mg venom) was similar to that recently reported using the plasma CK assay as an estimator of myonecrosis (Gutiérrez et al., 1991). The MTT-based method described here should be useful in the evaluation of the antimyotoxic potency of antivenoms, or in the screening of other neutralizing agents, such as monoclonal antibodies (Lomonte et al., 1992), or natural products (Mors et al., 1989).

Acknowledgements

We appreciate the help of Dr. Pedro Morera, Guillermo León, Rodrigo Chaves, Jorge Sanabria, and the serpentarium staff of the Instituto Clodomiro Picado. We also acknowledge the financial support of Vicerrectoría de Investigación (741-90-049), International Foundation for Science (F/1388-2 and F/0883-3), and the BID-CONICIT fellowship program. B.L. and J.M.G were recipients of a research career award from the Costa Rican National Scientific and Technological Research Council during this study.

References

Arce, V. (1986) A comparative histochemical study of bulbourethral and urethral glands in five rodent species. PhD Thesis. University of Cambridge, UK.

Bolaños, R. and Cerdas, L. (1980) Producción y control de sueros antiofídicos en Costa Rica. Bol. Of. Sanit. Panam. 88, 189.

Denizot, F. and Lang, R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J. Immunol. Methods 89, 271.

Grabar, P. (1986) The historical background of immunology. In: D. Stites, J.D. Stobo and J.V. Wells (Eds.), Basic and Clinical Immunology. Appleton and Lange, Norwalk, p. 3.

Gutiérrez, J.M., Chaves, F., Rojas, E. and Bolaños, R. (1980) Efectos locales inducidos por el veneno de la serpiente coral *Micrurus nigrocinctus* en ratón blanco. Toxicon 18, 633.

Gutiérrez, J.M., Arroyo, O., Chaves, F., Lomonte, B. and Cerdas, L. (1986) Pathogenesis of myonecrosis induced by coral snake (*Micrurus nigrocinctus*) venom in mice. Br. J. Exp. Pathol. 67, 1.

Gutiérrez, J.M., Rojas, G., Pérez, A., Argüello, I. and Lomonte, B. (1991) Neutralization of the venom of coral snake *Micrurus nigrocinctus* by a monovalent antivenom. Braz. J. Med. Biol. Res. 24, 701.

Hansen, M.B., Nielsen, S.E. and Berg, K. (1989) Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J. Immunol. Methods 119, 203.

Harris, J.B. and Cullen, M.J. (1990) Muscle necrosis caused by snake venoms and toxins. Electron Microsc. Rev. 3, 183.

Kouyoumdjian, J.A., Harris, J.B. and Johnson, M.A. (1986) Muscle necrosis caused by the subunits of crotoxin. Toxicon 24, 575.

Labbe, R., Lindsay, T., Gatley, R., Romaschin, A., Mickle, D., Wilson, G., Houle, S. and Walker, P. (1988) Quantitation of postischemic skeletal muscle necrosis: histochemical and radioisotope techniques. J. Surg. Res. 44, 45.

Lomonte, B., Gutiérrez, J.M., Ramírez, M. and Díaz, C. (1992) Neutralization of myotoxic phospholipases A_2 from the venom of the snake *Bothrops asper* by monoclonal antibodies. Toxicon 30, 239.

McLoon, L.K., Bauer, G. and Wirtschafter, J. (1991) Quantification of muscle loss in the doxorubicin-treated orbicularis oculi of the monkey. Effect of local injection of doxorubicin into the eyelid. Invest. Ophtalmol. Visual Sci. 32, 1667.

Mebs, D. and Ownby, C.L. (1990) Myotoxic components of snake venoms: their biochemical and biological activities. Pharmacol. Ther. 48, 223.

Mebs, D., Ehrenfeld, M. and Samejima, Y. (1983) Local necrotizing effect of snake venoms on skin and muscle: relationship to serum creatine kinase. Toxicon 21, 393.

Mors, W.B., Do Nascimento, M.C., Parente, J.P., Da Silva, M.H., Melo, P.A. and Suarez-Kurtz, G. (1989) Neutralization of lethal and myotoxic activities of South American rattlesnake venom by extracts and constituents of the plant *Eclipta prostrata* (Asteraceae). Toxicon 27, 1003.

Mosmann, T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55.

Nakada, F., Uezu, N., Ohshiro, M. and Miyagi, K. (1980) Relation between serum creatine phosphokinase activity and the amount of Habu (*Trimeresusrus flavoviridis*) snake venom injected in thigh muscle in rabbits. Toxicon 18, 351.

Nakada, K., Nakada, F., Ito, E. and Inoue, F. (1984) Quantification of myonecrosis and comparison of necrotic activity of snake venoms by determination of creatine phosphokinase activity in mice sera. Toxicon 22, 921.

Ownby, C.L., Gutiérrez, J.M., Colberg, T.R. and Odell, G.V. (1982) Quantitation of myonecrosis induced by myotoxin a from prairie rattlesnake (*Crotalus viridis viridis*) venom. Toxicon 20, 877.

Preston, S., Davis, C.E. and Harris, J.B. (1990) The assessment of muscle fibre loss after the injection of the venom of *Notechis scutatus* (Australian tiger snake). Toxicon 28, 201.

Suarez-Kurtz, G. (1982) Release of creatine kinase from frog muscle by osmotic changes. Am. J. Physiol. 242, C398.

World Health Organization (1981) Progress in the Characterization of Venoms and Standardization of Antivenoms. Geneva, WHO Offset Publication no. 58.

IV

Toxicon, Vol. 32, No. 1, pp. 41–55, 1994.
Printed in Great Britain.

0041-0101/94 $6.00 + .00

THE DYNAMICS OF LOCAL TISSUE DAMAGE INDUCED BY *BOTHROPS ASPER* SNAKE VENOM AND MYOTOXIN II ON THE MOUSE CREMASTER MUSCLE: AN INTRAVITAL AND ELECTRON MICROSCOPIC STUDY

BRUNO LOMONTE,[1,2]* JONAS LUNDGREN,[3] BENGT JOHANSSON[3] and ULF BAGGE[3]

[1]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; and Departments of [2]Clinical Immunology, and [3]Anatomy, University of Göteborg, Guldhedsgatan S-413 46 Göteborg, Sweden

(*Received* 14 *June* 1993; *accepted* 24 *August* 1993)

B. LOMONTE, J. LUNDGREN, B. JOHANSSON and U. BAGGE. The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. *Toxicon* **32,** 41–55, 1994.—The acute tissue damaging effects of *Bothrops asper* snake venom and a myotoxic Lys-49 phospholipase A_2 (myotoxin II) on the mouse cremaster muscle were studied by intravital and electron microscopy. Both venom and myotoxin induced local contractions of the muscle fibres within 10–60 sec after exposure, which disappeared after 1–2 min. This observation is consistent with the hypothesis that *Bothrops* myotoxins act initially at the sarcolemma by affecting its permeability and allowing an influx of calcium. The venom also induced an early but transient vasoconstriction of arterioles. The development of edema was monitored using i.v. FITC-dextran as a marker. Plasma leakage started after about 2 min of exposure to venom or myotoxin, was extensive by 4–5 min, and originated from small venules and their adjoining capillary segments. The venom induced formation of thrombi and emboli in venules, but not in arterioles. Haemorrhage appeared after 4–6 min of exposure, the bleedings always originating from capillaries and small venules. The microbleedings were explosive, appearing as rapid bursts of erythrocytes into the extravascular space, and suggesting a *per rhexis* type of haemorrhage. This was confirmed by electron microscopy evaluation of the same microvessels observed intravitally, which showed erythrocyte extravasation through gaps in damaged endothelial cells. Other phenomena in the microcirculation included blood-flow disturbances, crenation and sphering of erythrocytes, and stasis with dense packing of cells in capillary networks. Muscle necrosis, caused by either venom or myotoxin, started 3–4 min after application. The first sign of damage in the fibres was the development of a narrow, transverse band with local loss of striation. This was followed by slow retraction of myofibrils until there was a complete transverse rupture of the fibre. This process was often repeated along the same fibre, leaving a row of fragments separated by spaces apparently devoid of myofibrillar material. The results confirm the rapid tissue

* Author to whom correspondence should be addressed, at the University of Göteborg.

damaging effects of *B. asper* venom, implying that potentially useful blocking agents must be administered early and have the ability to diffuse rapidly into the tissues.

INTRODUCTION

ENVENOMATION due to snakebite is a health problem, especially in tropical regions of the world. In Latin America, most snakebites are caused by *Bothrops* spp. (ROSENFELD, 1971; CARDOSO, 1985). Their venoms, as typical of crotalids (OWNBY, 1990), induce a prominent local tissue damage with myonecrosis, haemorrhage, and oedema (GUTIÉRREZ and LOMONTE, 1989). *Bothrops asper* is the most frequent species causing snakebite poisoning in Central America (BOLAÑOS, 1984). The tissue-damaging effects of its venom have been studied experimentally by histological, immunohistochemical and biochemical methods (for a review, see GUTIÉRREZ and LOMONTE, 1989). However, the exact sequence and characteristics of the events occurring immediately after the contact between venom and tissue are not fully known.

In the present investigation, intravital and electron microscopy were combined to analyse the effects of local application of venom and a purified myotoxic component, myotoxin II (LOMONTE and GUTIÉRREZ, 1989), on the mouse cremaster muscle and its microvasculature.

MATERIALS AND METHODS

Cremaster preparation

Male C57BL mice (ALAB, Sweden), weighing 18–22 g, were anaesthetised with an i.p. injection of sodium pentobarbital (2 mg/100 g body weight) and placed in supine position on a water-heated bed. Intravenous injections were performed through the left femoral vein, which was exposed and cannulated under a stereo microscope by inserting a few mm of 33 gauge needle; 5–6 mm of the sharp end of a Hamilton needle was broken off and connected to a thin silicone elastomer tubing. The needle was secured in the vein by two 6–0 stitches into the thigh muscle.

To expose the cremaster muscle, the scrotum was stretched out and opened by cutting it across 3–4 mm from the tip. Through the hole, either the left or right cremasteric pouch was gently pulled out and its tip pinned onto an observation platform (Fig. 1). The pouch was opened longitudinally with micro-scissors and the mesoepididymis was divided so that the testicle and epididymis could be pushed away into the inguinal canal. The muscle

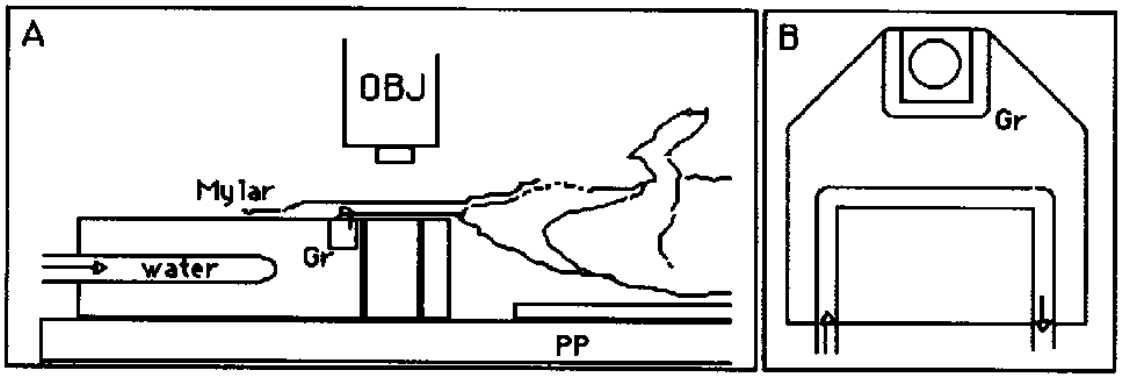

FIG. 1. (A) SIDE-VIEW OF THE PLATFORM USED FOR INTRAVITAL MICROSCOPY, WITH THE MOUSE RESTING ON ITS HEATED BED ON TOP OF A PLEXIGLASS PLATE (PP).
The cremaster muscle is spread over a circular transparent silicone cylinder, the muscle is pinned down at the edge on a silicone filled groove (Gr), and the surface is covered by a thin sheet of Mylar®; OBJ, objective. (B) Top-view of the brass block showing the circular observation area, the silicone-filled groove and water channels (arrows).

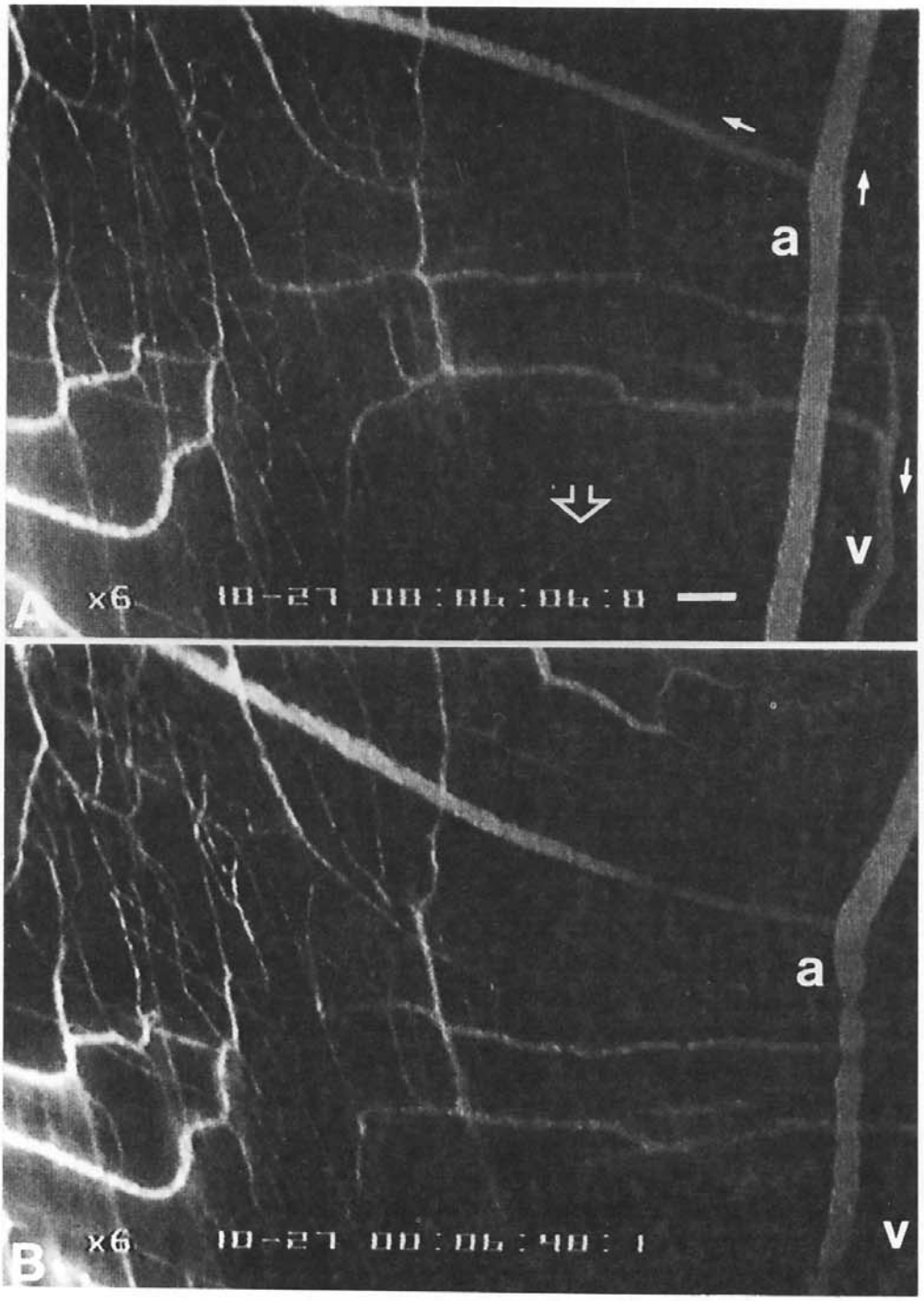

FIG. 2. MICROCIRCULATION VISUALISED WITH FITC-DEXTRAN 30 SEC AFTER EXPOSURE TO VENOM (A). After about 1 min of exposure to venom (B), muscle contractions severely distort the tissue (in direction of open arrow, shown in A) and its microvasculature. Sphincter-like constrictions are seen in an arteriole (a); v, venule. Arrows indicate directions of flow. Bar 100 μm.

was then spread out over the platform, which consisted of a transparent silicone elastomer (Rhodorsil RTV 141; Rhône-Poulenc, France) that was moulded into a hole (diam. 6 mm) drilled through a 10 mm thick block of brass. A silicone-filled groove in the brass block around the observation area was used to pin down the edges of the muscle with micro-staples. Heated water was circulated through the brass block to warm the silicone platform and maintain the muscle at 37°C. During the whole preparation the tissues were kept moist with warm saline. Finally, a thin Mylar® sheet (6 μm) was placed on top of the muscle to prevent evaporation.

Vital microscopy

The muscle preparation was observed in a Leitz vital microscope, equipped with a colour TV-camera (WV CD130; Panasonic, Japan), a black and white low light-level TV-camera for fluorescence microscopy (WV-1900/G; Panasonic), a VCR (NV-FS88 HQ; Panasonic), a video timer (VTG-33; FOR-A Co. Ltd, Japan), a 19″ video colour monitor (Panasonic), a VCR image enhancer (Detailer II; Vidicraft, Japan), and a video copy processor (P66E, Mitsubishi, Japan).

Leitz dry objectives ×2.5;0.08, ×6;0.18, ×20;0.32 and water immersion objectives ×55;084 and ×75;1.0 were used in combination with ×12.5 eye-pieces. A Leitz Ploemopak illuminator and a Xenon burner (150 W) were used for incident light fluorescence microscopy. This was employed to study microvascular plasma leakage, which was visualised by i.v. injection of 0.10–0.15 ml of FITC-dextran (25 mg/ml phosphate buffered saline; mol. wt 147,800; Sigma Chemical Co., U.S.A.) given 5 min before the local application of venom or myotoxin.

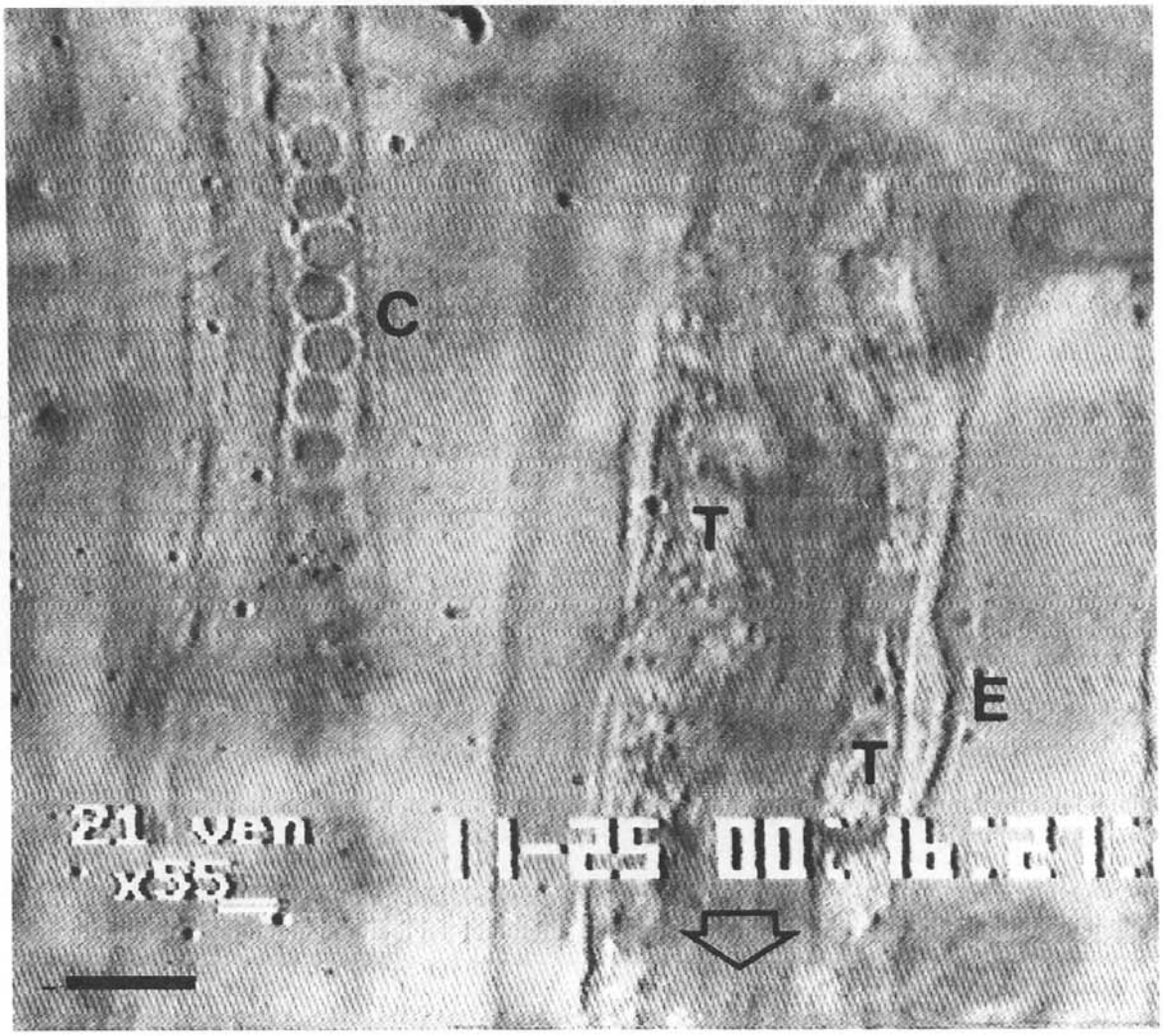

FIG. 3. EXAMPLE OF MICROVASCULAR FLOW DISTURBANCE DUE TO THROMBUS FORMATION (T) IN A SMALL VENULE AFTER EXPOSURE TO VENOM.

E, Endothelial cell nucleus; c, capillary containing a row of sphered erythrocytes. Open arrow indicates flow direction. Bar 10 μm.

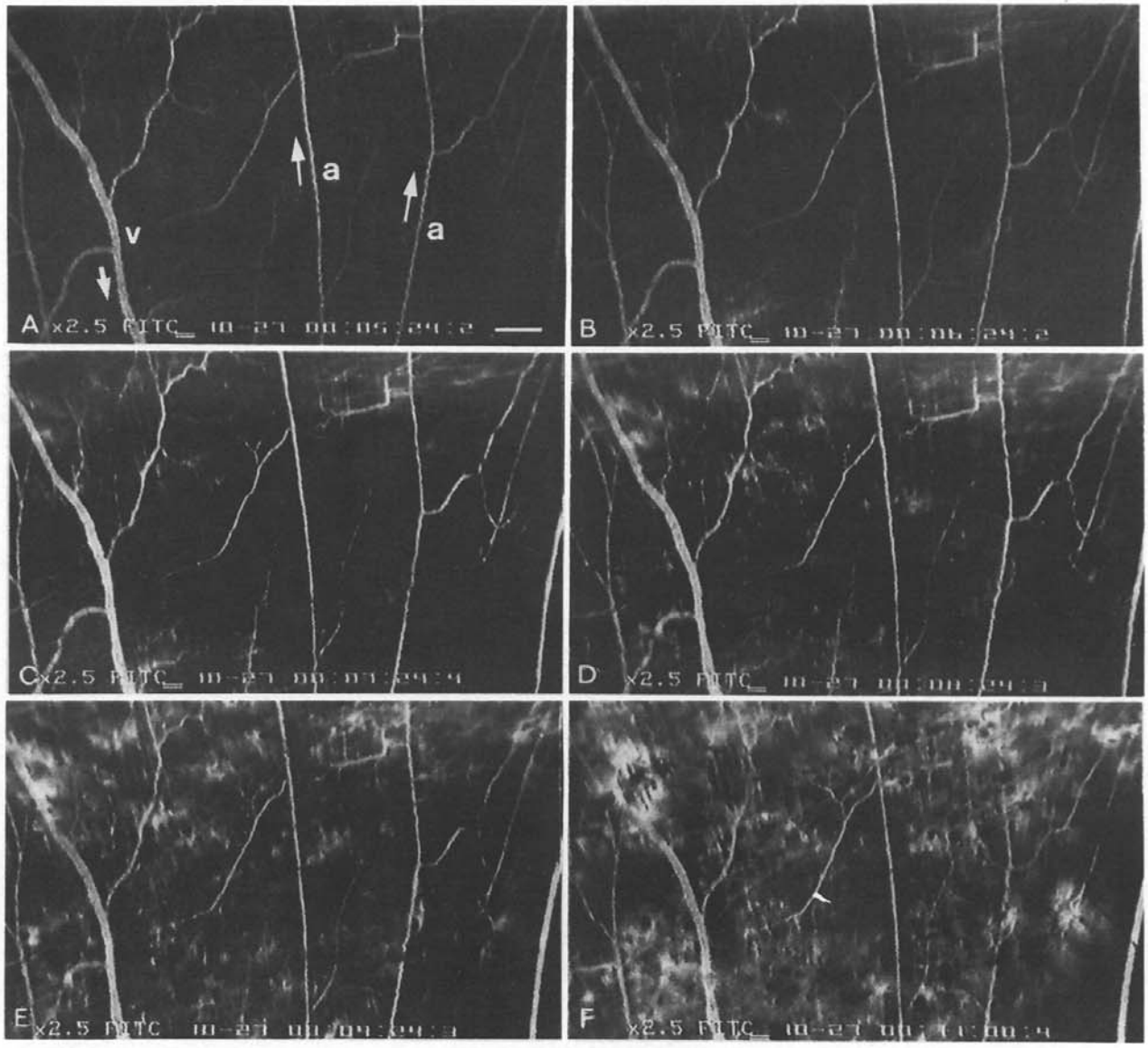

FIG. 4. DEVELOPMENT OF MICROVASCULAR LEAKAGE OF FITC-DEXTRAN.
(A) One minute after application of venom there are still no signs of leakage; (B–E) correspond to subsequent 1 min intervals; (F) 7 min after application of venom. a, Arteriole; v, venule. Arrows indicate flow directions. Bar 250 μm.

Ten cremaster muscles were exposed to venom and five muscles to myotoxin II. The venom was a pool obtained from more than 30 specimens of *B. asper* from the Pacific region of Costa Rica. Myotoxin II was purified from the venom as described by LOMONTE and GUTIÉRREZ (1989). This toxin is a lysine-49 phospholipase A_2 analogue (FRANCIS *et al.*, 1991), and is one of at least four biochemically and antigenically related isoforms found in this venom (LOMONTE and KAHAN, 1988), including myotoxins I (GUTIÉRREZ *et al.*, 1984*a*) and III (KAISER *et al.*, 1990).

For studies on plasma leakage, either venom or myotoxin were mixed with 1% agarose-saline at 50°C, and immediately 20 μl were spread over a small piece of transparent plastic film (Gelbond®; Pharmacia, Sweden) and cooled. The final concentration of the tested agents in the gel was 2.5 mg/ml. The thin layer of gel was gently placed on the muscle after moving the Mylar® to the side so that it covered about 2/3 of the muscle, serving as a control area. After 5 min exposure to the gel, the muscle was flushed with warm saline and covered with Mylar® again. The transparency of the gel allowed continuous observation of the microcirculation during the period that it was in contact with the muscle. For the rest of the experiments, the whole of the muscle was exposed to venom or myotoxin by lifting off the Mylar® and dripping 20 μl of a solution of either substance (2 mg/ml) on the muscle. The time of exposure varied from 5 to 30 min, during which the muscle was covered as described above, before being flushed with warm saline and once again covered for the following observation period (30 min).

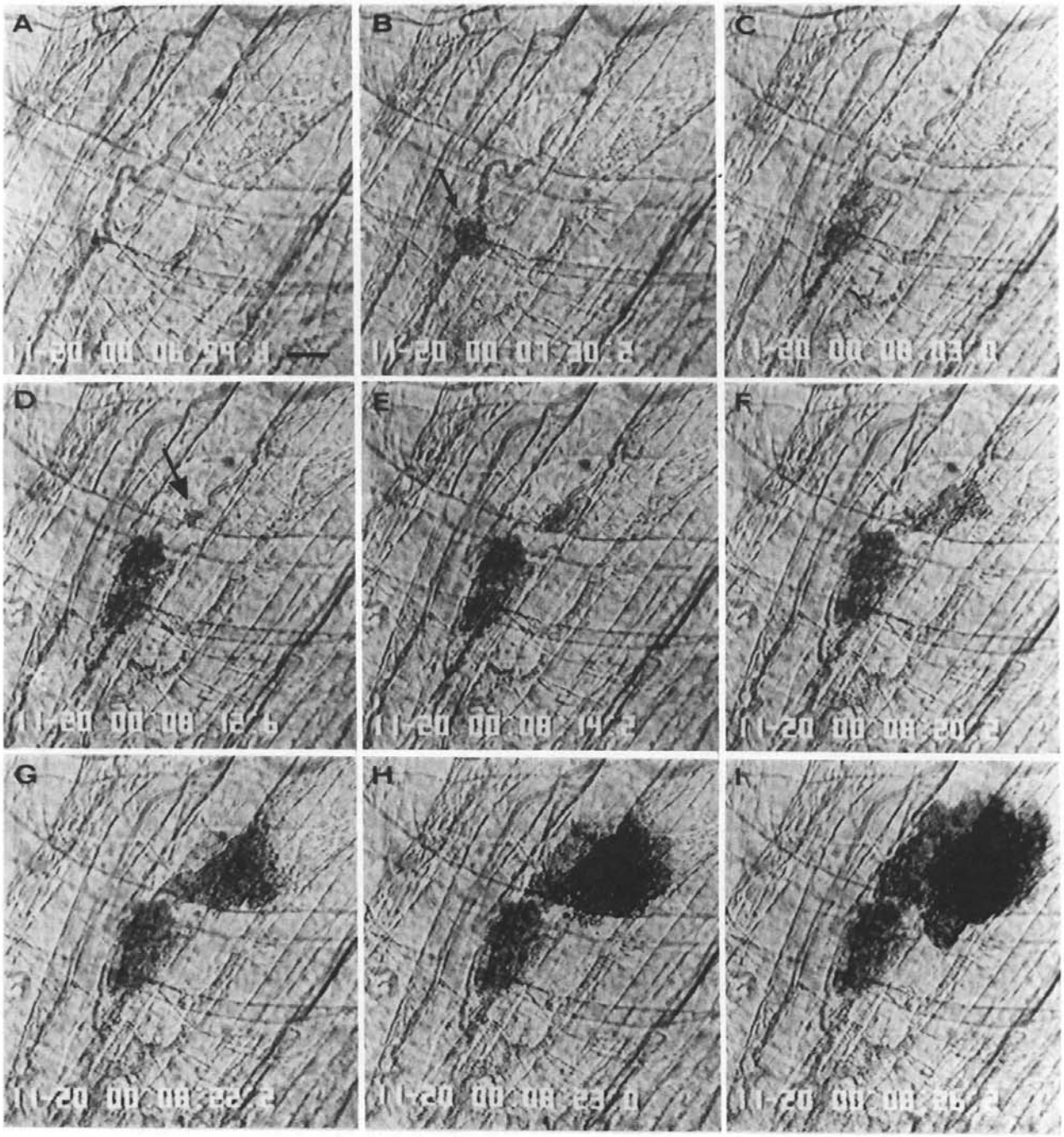

FIG. 5. SEQUENCE OF MICROPHOTOGRAPHS SHOWING THE DYNAMICS OF ERYTHROCYTE EXTRAVASATION AFTER EXPOSURE TO VENOM.

(A) After 5 min of exposure, there are no signs of vessel damage; the first bleeding is seen 30 sec later (at arrow in B), its size increasing at a moderate speed (C). A second, more explosive, bleeding erupts from a nearby capillary (arrow in D), reaching a considerable size in a few seconds (F–I). Bar 50 μm.

Electron microscopy (EM)

At the end of some experiments the cremaster muscle was fixed for 5 min with a solution consisting of 2.5% glutaraldehyde, 1% paraformaldehyde and 0.01% sodium azide in 0.05 M sodium cacodylate, pH 7.2, and then removed. The specimens were further fixed in the same solution overnight, followed by post-fixation in 1% OsO_4 in 0.1 M sodium cacodylate, dehydration and infiltration with epoxy resin (Agar 100) according to routine procedures. Ultrathin sections were cut with diamond knives in a Reichert Ultracut E microtome and contrasted with lead citrate and uranyl acetate before examination in a Zeiss CEM 902 electron microscope.

RESULTS

Venom

The first reactions seen in the vital microscope, and occurring between 10 and 60 sec after contact between venom and tissue, were muscle fibre contractions, which caused a pronounced mechanical distortion of the microvasculature (Fig. 2). At the same time, sphincter-like arteriolar constrictions were observed, causing intermittent reduction in the blood flow in the depending capillary networks for about 1 min (Fig. 2). Both muscle contractions and arteriolar constrictions ceased within 2 min, not to reappear even if the muscle was exposed to venom for up to 30 min. Another early event, occurring within 1 min and continuing during the whole observation period, was the formation of thrombi, giving off numerous emboli, in the postcapillary venules (Fig. 3), and an accumulation of leukocytes along the venular endothelium.

Leakage of FITC-dextran appeared after about 2 min of venom exposure, and mainly seemed to originate from small venules and their adjoining capillary segments (Fig. 4). About 5–6 min after the application of the venom, a large proportion of the observed area showed diffuse leakage. Stasis in the microcirculation, with a dense packing of erythrocytes in capillaries and small venules, occurred at about the same time as the plasma leakage.

Microbleedings regularly appeared after about 4–6 min of exposure and were always explosive in character. They originated from capillaries and small venules, and often developed as a series of burst-like enlargements of the microhaematoma before stopping within about 10–20 sec (Figs 5, 6). In many cases the blood flow through a ruptured microvessel continued after the bleeding had stopped. No bleedings originated from arterioles. Electron microscopy revealed that seemingly intact and ruptured microvessels could be found adjacent to each other in a damaged tissue area (Fig. 7A). Furthermore, the EM showed that erythrocytes escaped through gaps in the endothelial wall, with indications that some cellular component was fragmented at that site (Fig. 7B). Another striking phenomenon in the microcirculation, noted both intravitally and by EM, was the accumulation of spherical, but apparently not haemolysed, erythrocytes (Figs 3, 7). In some instances, it was possible to observe how normal disk-shaped erythrocytes, arriving in a feeding arteriole, rapidly crenated and became spherical upon entry into a capillary branch (Fig. 8).

As dramatic in appearance as the bleedings, but often already starting after about 3–4 min, was the degeneration of the individual muscle fibres. The process started as a focal loss of striation seen as a narrow band across the entire width of the fibre, followed by the appearance of a small, wedge-shaped lesion at one edge of the fibre and a slow retraction of the myofibrils in opposite directions, which widened the gap until the fibre suddenly ruptured. This rupturing was often repeated along the same fibre so that in the end there was only a row of fragments separated by spaces, apparently devoid of myofibrillar material (Fig. 9). Despite this severe morphological alteration of the tissue,

there was still blood flow in apparently undamaged microvessels running along and across the affected muscle fibres.

Myotoxin II

Application of myotoxin II to the cremaster resulted in a similar, rapid reaction as with venom, i.e. there were muscle contractions and a concomitant distortion of the microvasculature, but there were no arteriolar constrictions. The microvascular permeability also increased, although to a lesser extent than with whole venom. No thrombi, emboli or bleedings were observed during the experiments. The main lasting effect was the drastic alteration of muscle fibres, which was, dynamically and morphologically, indistinguishable from that caused by whole venom (Fig. 9).

DISCUSSION

In the present investigation, the direct microscopic observation of the reactions occurring after venom exposure of the mouse cremaster muscle and its microcirculation, made it possible to determine the sequence of events, in addition to their morphological characteristics, *in vivo*. The earliest detectable reaction was a muscle contraction response, occurring within seconds after exposure to the venom. The same reaction was observed when purified myotoxin II was applied, implying that this effect is due, at least in part, to the action of venom myotoxins. No response was observed when control gels with saline alone were applied onto the tissue preparations, demonstrating that this was a specific effect of the tested agents. The rapid appearance of muscle fibre contractions is consistent with the hypothesis that *Bothrops* myotoxins act initially on the sarcolemma, altering the permeability of the membrane and allowing an influx of calcium to the cytosol (GUTIÉRREZ *et al.*, 1984*a*, *b*; BRENES *et al.*, 1987; DIAZ *et al.*, 1991; RUFINI *et al.*, 1992; BULTRON *et al.*, 1993). However, other possible causes for this effect cannot be ruled out. Concomitant with the muscle contractions, reversible arteriolar constrictions were seen with whole venom, although not with myotoxin II. A similar transient vasoconstriction response of arterioles was described by OHSAKA (1979) using the venom of the Asian crotalid *Trimeresurus flavoviridis* on the rat mesentery.

Another early, but lasting, event (after exposure to venom, but not to myotoxin) was the formation of thrombi on the walls of the small venules, releasing emboli and disturbing the local blood flow. *Bothrops asper* venom has direct effects on the coagulation system *in vitro* (GENÉ *et al.*, 1989) and a thrombin-like enzyme has been isolated and characterised (ARAGON and GUBENSEK, 1978). *In vivo*, alterations of the coagulation system have been documented in patients (BARRANTES *et al.*, 1985) and in the mouse model (CHAVES *et al.*, 1989). It is likely that endothelial cell damage and activation, evidenced by the bleedings, oedema, and leukocyte adherence, also participate in the induction of the thrombotic events observed.

FIG. 6. SEQUENCE OF MICROPHOTOGRAPHS SHOWING AN ARTERIOLE (FLOW DIRECTION INDICATED BY LARGE OPEN ARROW) AND A CAPILLARY (SMALL OPEN ARROW).
In (A) erythrocytes are seen to tumble at the edge of the blood stream in the arteriole but no blood cells flow into the capillary until, suddenly, erythrocytes (e) rapidly stream into the capillary and through a small opening (arrow in B) in the endothelial wall (B–F). Bar 10 μm.

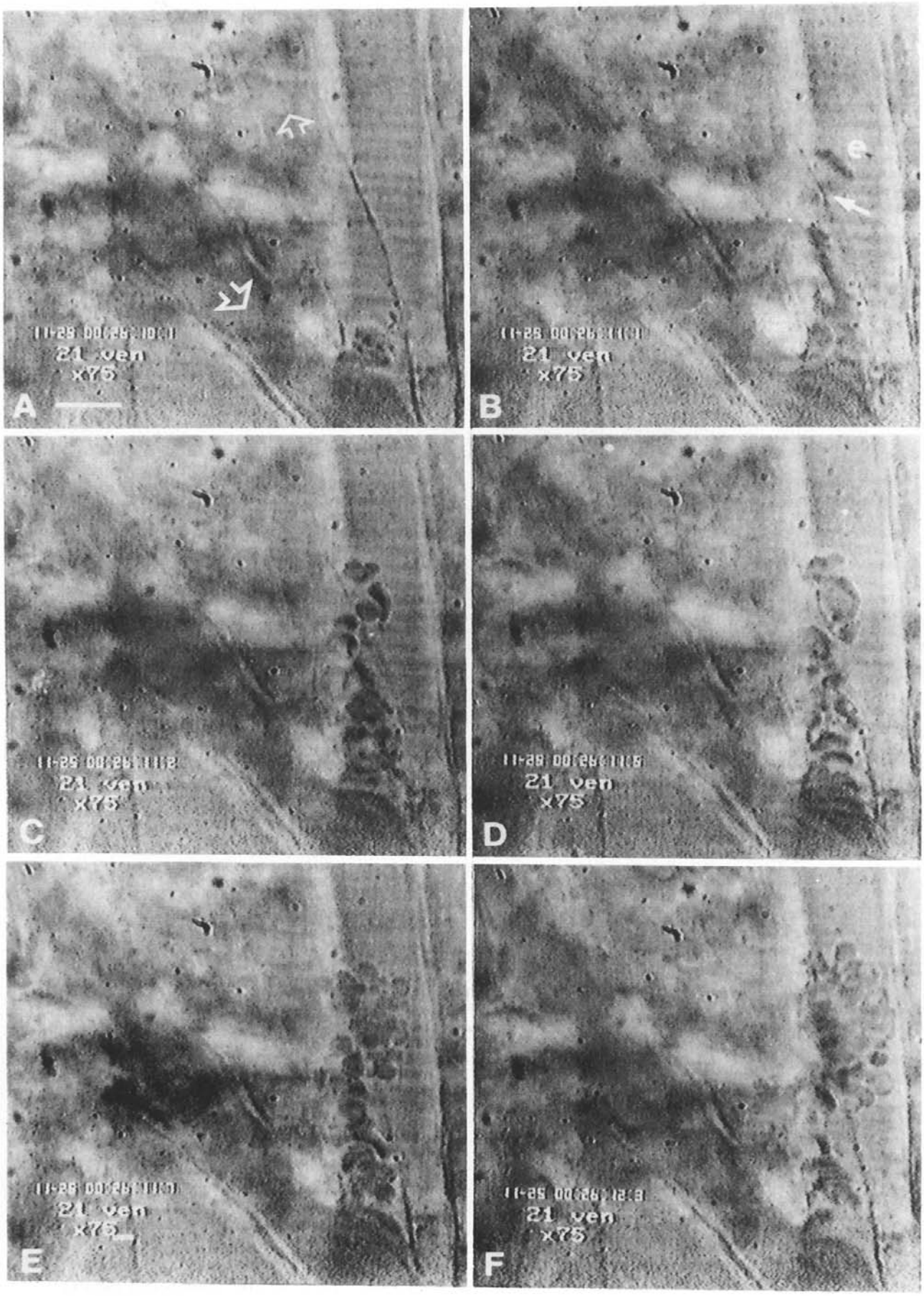
21 ven
x75
A
21 ven
x75
B
21 ven
x75
C
21 ven
x75
D
21 ven
x75
E
21 ven
x75
F

FIG. 6

Bothrops asper venom is known to induce a marked oedema response (GUTIÉRREZ *et al.*, 1980; LOMONTE *et al.*, 1993). Using FITC-dextran, it could be determined that both venom and myotoxin II rapidly increased vascular permeability, especially at the level of small venules and their adjoining capillary segments. The leakage induced by myotoxin was of a lower magnitude than that caused by whole venom, in agreement with earlier characterisation of its pharmacological activities (LOMONTE and GUTIÉRREZ, 1989). The mechanism of oedema induction by myotoxin II is not known, and cannot be attributed to phospholipase A_2 activity since it is a lysine-49 analogue (FRANCIS *et al.*, 1991). One possibility is that oedema develops as a response to muscle fibre necrosis. Alternatively, there could be a direct action of myotoxin on endothelial cells. This is currently being investigated.

When a widespread FITC-dextran leakage had developed, i.e. after about 5–6 min of exposure to venom, stasis in the microcirculation was also evident, together with erythrocyte sphering and packing. It is reasonable to assume that stasis was at least partly due to the significant plasma loss to the interstitial space, causing local haemoconcentration, and to the shape changes of the erythrocytes, altering their rheological properties. The mechanisms responsible for the erythrocyte sphering *in vivo* are not known, but in a simple test *in vitro*, we saw an immediate crenation and sphering of mouse erythrocytes after mixing them with whole venom, which suggests that the phenomenon may be due to a direct action of venom components on the erythrocyte membrane. QUIROS *et al.* (1992) recently described a moderate haemolytic effect of *B. asper* venom at high concentrations on mouse erythrocytes *in vitro*, although morphological alterations were not analysed. Sphering of erythrocytes *in vivo* was also observed after injection of *Vipera palestinae* venom (DANON *et al.*, 1961) and a phospholipase isolated from it (MCKAY *et al.*, 1970).

After at least 4 min of contact with the venom, bleedings started to appear from microvessels. These events were explosive in character, suggesting that the type of haemorrhage caused by this venom is *per rhexis* rather than *per diapedesis*, the latter mechanism being observed by intravital microscopy in the case of venoms from *T. flavoviridis* (OHSAKA, 1979), one of its purified haemorrhagic toxins (OSHIO, 1984), and *Agkistrodon bilineatus* (reviewed by OHSAKA, 1979). In the present study, electron microscopy of the same vessels that were observed in the vital microscope indicated that erythrocytes passed through minute vessel wall perforations. Although at the two-dimensional level it is not possible to exclude with certainty that the endothelial gap formation was the result of an opening of the junctions, the presence of cell debris at the site of the gap strongly suggests the formation of a transcellular opening, with a true local destruction of the vessel wall. This is also in agreement with the dynamics of the microbleedings observed intravitally. Thus, our findings support the results presented by MOREIRA *et al.* (1992) using *B. asper* venom. Isolated haemorrhagic factors from the venoms of *Crotalus*

FIG. 7. (A) ELECTRON MICROGRAPH OF THE SAME AREA AS IN FIG. 6, SHOWING EXTRAVASATED, ABNORMALLY ROUNDED ERYTHROCYTES IN AND AROUND A SMALL VENULE (ARROWHEADS). One erythrocyte (asterisk) is passing through the vessel wall. A normal small venule (V) containing normally shaped erythrocytes, and normal (N) and damaged (D) muscle fibres appear in the same field (×1900). (B) Magnification showing the passage of the erythrocyte (asterisk) through a small gap in the wall. This vessel shows a poor definition of the membrane of the endothelial cell (e) and of the basal lamina (arrows). At the acute angle formed by the erythrocyte there is obviously some cell debris, indicating a cellular fragmentation (of unknown origin) at this spot (open arrow) (×25,000).

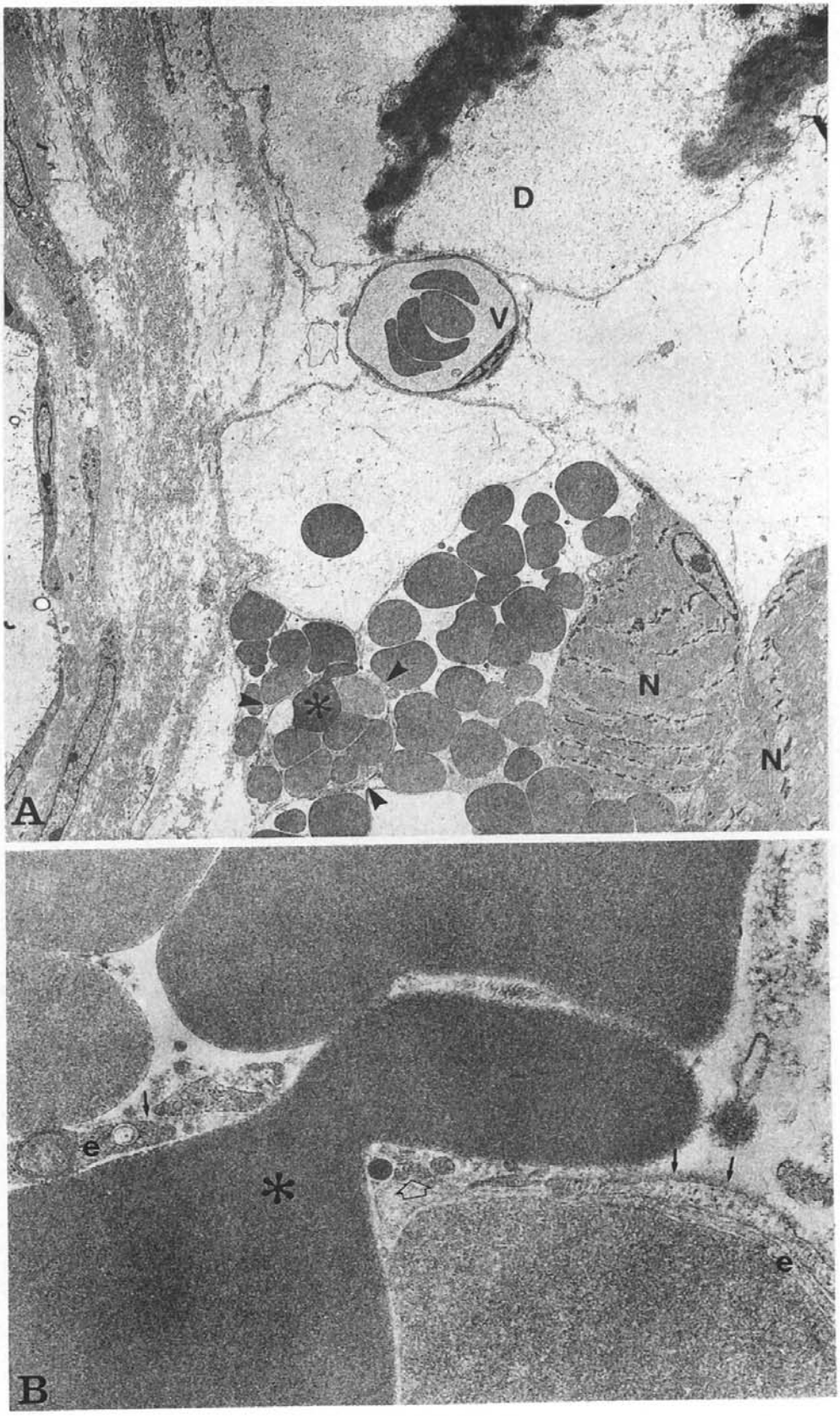

FIG. 7

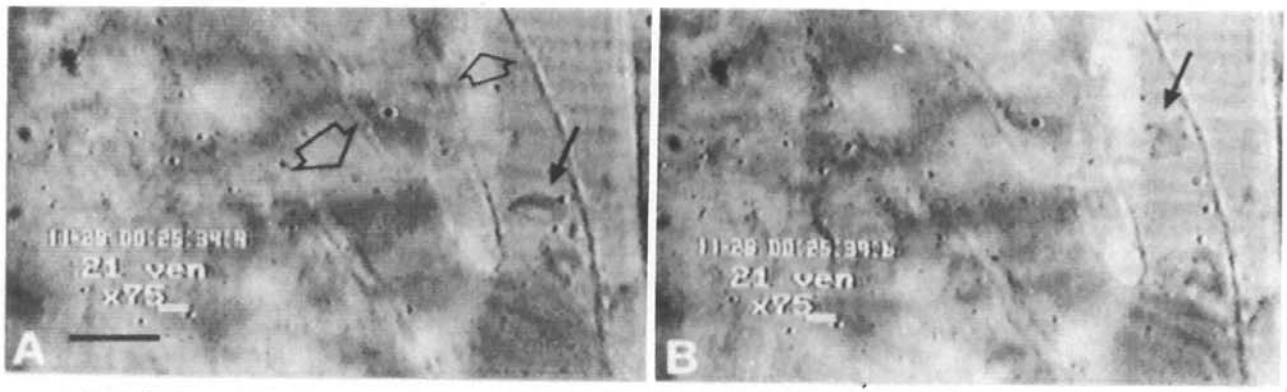

FIG. 8. THE SAME VESSELS AS IN FIG. 5, BEFORE THE BLEEDING OCCURRED, SHOWING AN EXAMPLE OF ERYTHROCYTE CRENATION.
A morphologically normal erythrocyte (arrow) leaving the feeding arteriole (big open arrow) (A) becomes crenated within a few seconds after entering the capillary side branch (small open arrow) (B). Bar 10 μm.

atrox, *C. horridus horridus*, and *A. bilineatus* have also been shown to induce a *per rhexis* type of erythrocyte extravasation (reviewed by OWNBY, 1990; OWNBY *et al.*, 1978, 1990).

Simultaneously with the bleedings, a series of striking morphological alterations occurred in individual muscle fibres. The first event appeared as a focal transverse loss of striation, followed by retraction and rupture of the fibres. Interestingly, at a relatively short distance from cellular rupture sites, normal striations were still visible, until the process also spread into these areas. Therefore, muscle fibre damage induced by either venom or myotoxin II does not develop as a homogeneous degeneration of the whole fibre, but has a markedly focal character, slowly progressing along the individual fibres. The initial stages of muscle fibre rupture appeared similar to delta lesions, described earlier in Duchenne muscular dystrophy (MOKRI and ENGEL, 1975) and after injection of myotoxins from *Bothrops* venoms (GUTIÉRREZ *et al.*, 1984*b*, 1991). Previous studies with *B. asper* venom and myotoxins have shown, by electron microscopy, that hypercontracted muscle fibres simultaneously display alterations which are indicative of irreversible cell damage (GUTIÉRREZ *et al.*, 1984*b*). Thus, the early morphological events described in this report correspond to the initial stages of the rapid myonecrosis process caused by this venom.

Using the same model and with the present results as a basis, future studies will focus on the search for agents with the ability to block or modify the acute venom actions *in vivo*. As shown by this study, such agent(s) must be administered very quickly after envenomation and diffuse rapidly into the tissues to reduce tissue damage efficiently.

FIG. 9. SEQUENCE OF MICROPHOTOGRAPHS SHOWING THE DYNAMICS OF MUSCLE FIBRE DAMAGE.
In (A) the muscle fibre (M) has already one rupture (at arrow 1), and a second transverse rupture is starting (arrow 2). In (B), 16 sec later, the transverse lesion has evolved into a frank rupture on one side. The retraction and rupture of the fibre continues in (C) while a third lesion already appears in (D) (arrow 3). Before the third rupture is complete, the space created by retraction of the muscle fibre is filled with a bubble-like material, presumably intercellular fluid (E, F). A fourth rupture (arrow 4) appears in (G). (H) Shows the same area, with the muscle fibre fragments and spaces, at lower magnification. Bar 10 μm.

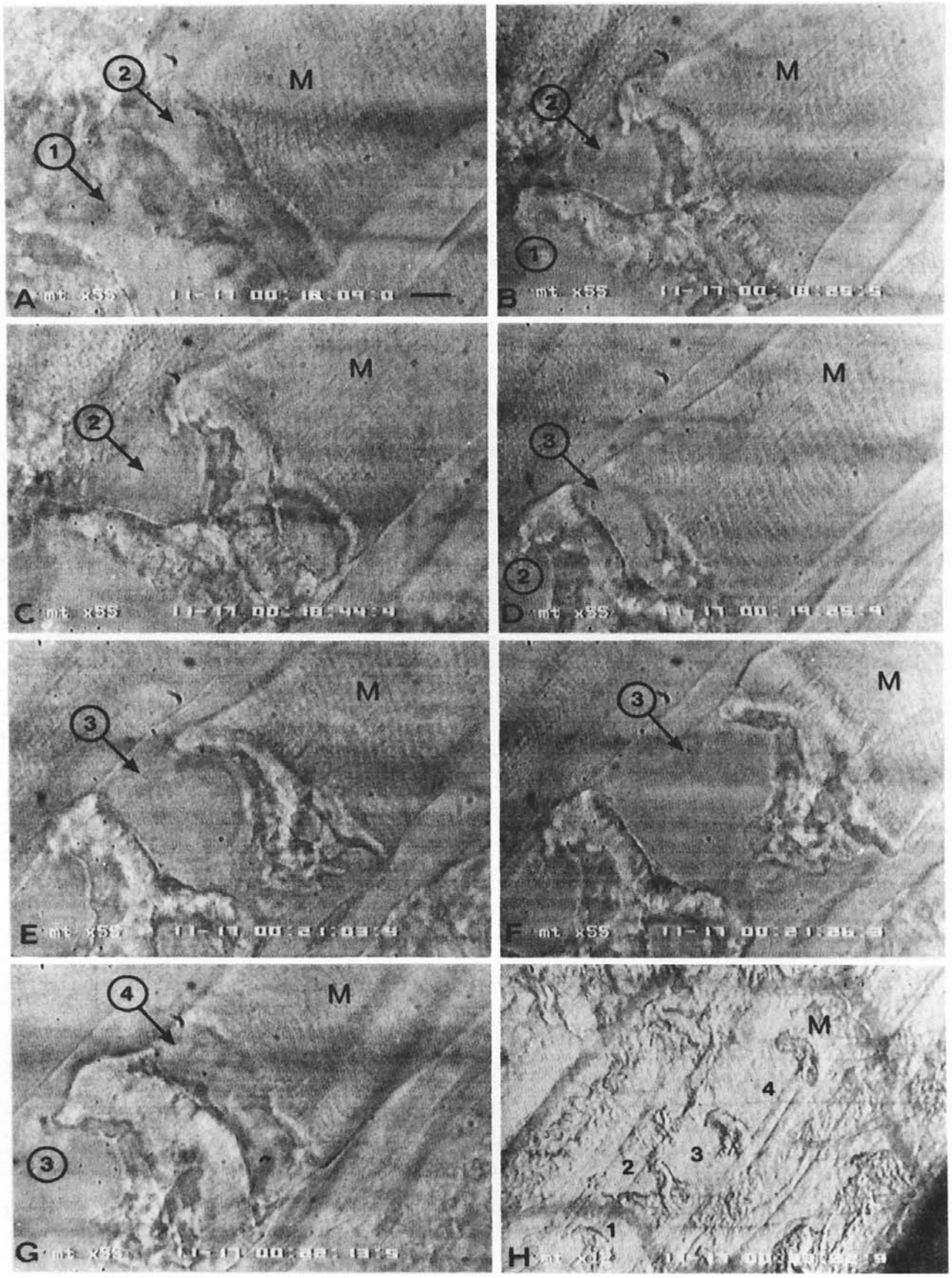

FIG. 9

Acknowledgements—This work was performed at the Department of Anatomy, University of Göteborg, and supported by the Swedish Medical Research Council (00663), the Swedish Agency for Research Cooperation with Developing Countries (SAREC), and Consejo Nacional de Investigaciones Científicas y Tecnológicas de Costa Rica (CONICIT). We thank Dr J. M. GUTIÉRREZ for constructive criticism during this work.

REFERENCES

ARAGON, F. and GUBENSEK, F. (1978) Characterization of thrombin-like proteinase from *Bothrops asper* venom. In: *Toxins: Animal, Plant and Microbial*, pp. 107–111 (ROSENBERG, P., Ed.). Oxford: Pergamon Press.

BARRANTES, A., SOLIS, V. and BOLAÑOS, R. (1985) Alteración de los mecanismos de la coagulación en el envenenamiento por *Bothrops asper* (terciopelo). *Toxicon* **23**, 399–407.

BOLAÑOS, R. (1984) *Serpientes, Venenos y Ofidismo en Centroamérica*, 136 pp. San José: Editorial Universidad de Costa Rica.

BRENES, F., GUTIÉRREZ, J. M. and LOMONTE, B. (1987) Immunohistochemical demonstration of the binding of *Bothrops asper* myotoxin to skeletal muscle sarcolemma. *Toxicon* **25**, 574–577.

BULTRON, E., GUTIÉRREZ, J. M. and THELESTAM, M. (1993) Effects of *Bothrops asper* (terciopelo) myotoxin III, a basic phospholipase A_2, on liposomes and mouse gastrocnemius muscle. *Toxicon* **31**, 217–222.

CARDOSO, J. L. C. (1985) Snakebites at hospital Vital Brazil. A study of 2908 cases. *Toxicon* **23**, 558.

CHAVES, F., GUTIÉRREZ, J. M., LOMONTE, B. and CERDAS, L. (1989) Histopathological and biochemical alterations induced by intramuscular injection of *Bothrops asper* (terciopelo) venom in mice. *Toxicon* **27**, 1085–1093.

DANON, D., GITTER, S. and ROSEN, M. (1961) Deformation of red cell shape induced by *Vipera palestinae* venom *in vivo*. *Nature* **189**, 320–321.

DIAZ, C., GUTIÉRREZ, J. M., LOMONTE, B. and GENÉ, J. A. (1991) The effects of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2 anticoagulant and myotoxic activities. *Biochim. biophys. Acta* **1070**, 455–460.

FRANCIS, B., GUTIÉRREZ, J. M., LOMONTE, B. and KAISER, I. (1991) Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A_2. *Archs Biochem. Biophys.* **284**, 352 359.

GENÉ, J. A., ROY, A., ROJAS, G., GUTIÉRREZ, J. M. and CERDAS, L. (1989) Comparative study on coagulant, defibrinating, fibrinolytic and fibrinogenolytic activities of Costa Rican crotaline snake venoms and their neutralization by a polyvalent antivenom. *Toxicon* **27**, 841 848.

GUTIÉRREZ, J. M. and LOMONTE, B. (1989) Local tissue damage induced by *Bothrops* snake venoms. A review. *Mem. Inst. Butantan* **51**, 211–223.

GUTIÉRREZ, J. M., ARROYO, O. and BOLAÑOS, R. (1980) Mionecrosis, hemorragia y edema inducidos por el veneno de *Bothrops asper* en ratón blanco. *Toxicon* **18**, 603–610.

GUTIÉRREZ, J. M., OWNBY, C. L. and ODELL, G. V. (1984*a*) Isolation of a myotoxin from *Bothrops asper* venom: partial characterization and action on skeletal muscle. *Toxicon* **22**, 115–128.

GUTIÉRREZ, J. M., OWNBY, C. L. and ODELL, G. V. (1984*b*) Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Expl mol. Path.* **40**, 367–379.

GUTIÉRREZ, J. M., NUÑEZ, J., DIAZ, C., CINTRA, A. C. O., HOMSI-BRANDEBURGO, M. I. and GIGLIO, J. R. (1991) Skeletal muscle degeneration and regeneration after injection of bothropstoxin II, a phospholipase A_2 isolated from the venom of the snake *Bothrops jararacussu*. *Expl mol. Path.* **55**, 217–229.

KAISER, I. I., GUTIÉRREZ, J. M., PLUMMER, D., AIRD, S. and ODELL, G. V. (1990) The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. *Archs Biochem. Biophys.* **278**, 319–325.

LOMONTE, B. and GUTIÉRREZ, J. M. (1989) A new muscle-damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27**, 725–733.

LOMONTE, B. and KAHAN, L. (1988) Production and partial characterization of monoclonal antibodies to *Bothrops asper* (terciopelo) myotoxin. *Toxicon* **26**, 675–689.

LOMONTE, B., TARKOWSKI, A. and HANSON, L. Å. (1993) Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* **17**, 93–105.

MCKAY, D. G., MOROZ, C., DE VRIES, A., CSAVOSSY, I. and CRUSE, V. (1970) The action of hemorrhagin and phospholipase derived from *Vipera palestinae* venom on the microcirculation. *Lab. Invest.* **22**, 387–399.

MOKRI, B. and ENGEL, A. G. (1975) Duchenne dystrophy: electron microscopic findings pointing to a basic or early abnormality in the plasma membrane of the muscle fiber. *Neurology* **25**, 1111–1120.

MOREIRA, L., GUTIÉRREZ, J. M., BORKOW, G. and OVADIA, M. (1992) Ultrastructural alterations in mouse capillary blood vessels after experimental injection of venom from the snake *Bothrops asper* (terciopelo). *Expl mol. Path.* **57**, 124–133.

OHSAKA, A. (1979) Hemorrhagic, necrotizing and edema-forming effects of snake venoms. In: *Handbook of Experimental Pharmacology*, Vol. 52, pp. 480–546 (LEE, C. Y., Ed.). New York: Springer.

OSHIO, C. (1984) The behaviour of platelets in capillary hemorrhage induced by snake venom. *Keio J. Med.* **33**, 83–97.

OWNBY, C. L. (1990) Locally acting agents: myotoxins, hemorrhagic toxins and dermonecrotic factors. In: *Handbook of Toxinology*, pp. 602–654 (SHIER, W. T. and MEBS, D., Eds). New York: Marcel Dekker.

OWNBY, C. L., BJARNASON, J. and TU, A. T. (1978) Hemorrhagic toxins from rattlesnake (*Crotalus atrox*) venom. Pathogenesis of hemorrhage induced by three purified toxins. *Am. J. Path.* **93,** 201–218.

OWNBY, C. L., NIKA, T., IMAI, K. and SUGIHARA, H. (1990) Pathogenesis of hemorrhage induced by bilitoxin, a hemorrhagic toxin isolated from the venom of the common cantil (*Agkistrodon bilineatus bilineatus*). *Toxicon* **28,** 837–846.

QUIROS, S., GENÉ, J. A., GUTIÉRREZ, J. M. and THELESTAM, M. (1992) Effect of *Bothrops asper* (fer-de-lance) snake venom on erythrocyte membrane. A comparative study. *Comp. Biochem. Physiol.* **101C,** 433–436.

ROSENFELD, G. (1971) Symptomatology, pathology and treatment of snake bites in South America. In: *Venomous Animals and their Venoms*, Vol. 2, pp. 345–403 (BUCHERL, W. and BUCKLEY, E. E., Eds). New York: Academic Press.

RUFINI, S., CESARONI, P., DESIDERI, A., FARIAS, R., GUBENSEK, F., GUTIÉRREZ, J. M., LULY, P., MASSOUD, R., MORERO, R. and PEDERSEN, J. Z. (1992) Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. *Biochemistry* **31,** 12,424–12,430.

V

Pergamon

Toxicon, Vol. 32, No. 4, pp. 505–510, 1994

0041-0101/94 $6.00 + 0.00

0041-0101(93)E0010-Q

SHORT COMMUNICATIONS

ACTIVITY OF HEMORRHAGIC METALLOPROTEINASE BaH-1 AND MYOTOXIN II FROM *BOTHROPS ASPER* SNAKE VENOM ON CAPILLARY ENDOTHELIAL CELLS *IN VITRO*

BRUNO LOMONTE,[1,3] JOSÉ MARÍA GUTIÉRREZ,[1] GADI BORKOW,[2] MICHAEL OVADIA,[2] ANDREJ TARKOWSKI[3,4] and LARS Å. HANSON[3]

[1]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; [2]George S. Wise Faculty of Life Sciences, Department of Zoology, Tel Aviv University, Tel Aviv, Israel; and [3]Departments of Clinical Immunology, and [4]Rheumatology, University of Göteborg, Sweden

(*Received* 3 *September* 1993; *accepted* 20 *October* 1993)

B. LOMONTE, J. M. GUTIÉRREZ, G. BORKOW, M. OVADIA, A. TARKOWSKI and L. Å. HANSON. Activity of hemorrhagic metalloproteinase BaH-1 and myotoxin II from *Bothrops asper* snake venom on capillary endothelial cells *in vitro*. *Toxicon* **32**, 505–510, 1994.—*In vivo*, hemorrhagic toxins isolated from snake venoms cause a disorganization of the basal lamina of capillaries, with a concomitant degenerative process of endothelial cells. In this study we investigated the effects of BaH-1, a hemorrhagic metalloproteinase purified from the venom of *Bothrops asper*, on a murine endothelial cell line of capillary origin. A quantitative cytotoxicity assay based on the release of lactic dehydrogenase was utilized. BaH-1, despite its potent hemorrhagic activity, did not exert direct cytolytic activity on the endothelial cells, even at concentrations as high as 65 μg/ml. The only visible effect of BaH-1 on the cultured cells was a relatively slow, moderate detachment of cells, interpreted as a consequence of proteolytic degradation of extracellular matrix components. In contrast, myotoxin II, a lysine-49 phospholipase A_2 from the same venom, was clearly cytotoxic to this cell type, albeit being devoid of hemorrhagic activity. These findings suggest that the ability of venom metalloproteinases to induce hemorrhage is not related to a direct cytotoxic action on endothelial cells, and that the rapid degenerative changes of endothelium observed *in vivo* are probably the result of an indirect mechanism.

TOXINS causing hemorrhage are widely distributed in venoms from snakes of the Crotalidae and Viperidae families (for a review, see BJARNASON and FOX, 1988–1989; OWNBY, 1990). During the last years, a number of hemorrhagic toxins have been isolated and biochemically characterized as metalloproteinases, which, despite differences in molecular weight, appear to constitute a related family of proteins arising from a single ancestral gene (HITE, *et al.*, 1992; PAINE *et al.*, 1992). The mode of action of these toxins is not completely understood. Several studies have shown their ability to proteolytically degrade, *in vitro*, major components of the basal lamina and extracellular matrix, such as laminin,

fibronectin, collagen type IV, entactin/nidogen, and gelatins (BJARNASON *et al.*, 1988; BARAMOVA *et al.*, 1989; MARUYAMA *et al.*, 1992; TAKACS *et al.*, 1992). However, studies of the possible direct actions of hemorrhagic toxins on endothelium are lacking. *In vivo* investigations of the hemorrhage induced by purified toxins have shown that, in addition to the disorganization of the basal lamina of capillaries, endothelial cells undergo a series of conspicuous degenerative morphological alterations which can progress until complete ruptures or gaps are formed within the cells (OWNBY, 1990; OWNBY and GEREN, 1978; OWNBY *et al.*, 1978, 1990). Therefore, the possibility of a direct action of hemorrhagic toxins on endothelial cells has been pointed out (OWNBY and GEREN, 1978; OWNBY *et al.*, 1990; GUTIERREZ and LOMONTE, 1989). As many factors may participate *in vivo*, it was necessary to address this point by using isolated endothelial cells growing *in vitro*.

In this study, the activity of BaH-1, a hemorrhagic toxin recently purified from the venom of *Bothrops asper* (BORKOW *et al.*, 1993), on cultured endothelial cells, was investigated. It has been previously shown that *B. asper* venom affects the integrity of capillaries in a mouse model (MOREIRA *et al.*, 1992). Furthermore, intravital microscopic observation of the hemorrhage process indicated that capillary-size microvessels are the main target of this venom, losing their integrity by 4–6 min after exposure (LOMONTE *et al.*, 1994). Therefore, a mouse endothelial cell line of capillary origin was utilized in these experiments.

Hemorrhagic toxin BaH-1 was purified as described by BORKOW *et al.* (1993), and its biological activity was verified just before the cytotoxicity experiments, by intradermal injection (2 μg) of mice according to the method of KONDO *et al.* (1960). Myotoxin II, from the same venom, was purified as described by LOMONTE and GUTIERREZ (1989), and it has been biochemically characterized as a lysine-49 phospholipase A_2 isoform (FRANCIS *et al.*, 1991). A murine endothelial cell line of capillary origin (tEnd), established by transformation with the polyoma virus middle T oncogene (BUSSOLINO *et al.*, 1991), was kindly provided by Dr A. MANTOVANI (Istituto Mario Negri, Milano, Italy). Cells were routinely grown in Iscove's medium (Gibco, Paisley, U.K.) supplemented with 10% fetal calf serum (FCS; Biological Ind., Haemek, Israel), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 0.05 mg/ml gentamycin, in a humidified atmosphere of 5% CO_2 and 95% air. In order to quantify cytotoxicity, cells were seeded at $1–4 \times 10^4$/well in 96-well plates and grown for 2–4 days. At the moment of the assay, culture medium was removed and substituted with 150 μl/well of medium with 1% FCS containing different amounts of toxin, and incubated for 3 hr at 37°C. Then, supernatant (100 μl) was assayed for lactate dehydrogenase (LDH) released from damaged cells (kit No. 500, Sigma Chemical Co., U.S.A.). The FCS concentration was lowered to 1% to minimize the basal activity of LDH in the medium. As controls for 100% and 0% cytotoxicity, cells were incubated with 0.1% Triton X-100-containing medium, or medium without toxin, respectively. Each sample was assayed in triplicate wells, in at least two independent experiments. To rule out a possible interference of BaH-1 in the LDH assay (due to its proteolytic activity), a control experiment was performed in which cell supernatants containing LDH activity were incubated with or without BaH-1, at the highest concentration utilized (65 μg/ml), for 3 hr at 37°C. Then, LDH activity of the samples was determined as described.

Toxin BaH-1, despite its potent hemorrhagic action *in vivo*, did not induce any significant cytotoxicity on the capillary endothelial cells in culture, even when concentrations as high as 65 μg/ml were tested (Fig. 1). The toxin per se did not interfere with the LDH assay utilized (data not shown). The main visible effect of BaH-1 toxin on endothelial cells was a relatively slow, moderate detachment, with some of the cells

becoming round (Fig. 2). This effect was dose-dependent, and can be interpreted as a consequence of the proteolytic digestion of extracellular matrix components, as it has been shown that BaH-1 is a metalloproteinase (BORKOW *et al.*, 1993). For comparison, myotoxin II, which does not induce hemorrhage (LOMONTE and GUTIERREZ, 1989), was clearly cytotoxic to the endothelial cells, under identical assay conditions (Fig. 1). These findings suggest that the hemorrhagic mechanism of BaH-1 is not related to a direct cytotoxic effect on endothelial cells. Thus, the degenerative changes of endothelial cells observed *in vivo*, after injection both of whole venom (MOREIRA *et al.*, 1992) and of purified BaH-1 (L. MOREIRA, G. BORKOW, M. OVADIA and J. M. GUTIÉRREZ, unpublished results) are probably the result of an indirect mechanism. ARAKI *et al.* (1993) recently reported evidence for the induction of apoptosis in cultured human endothelial cells after incubation with crude hemorrhagic snake venoms. However, their results are difficult to interpret due to the use of crude venoms, since, as shown in the present report, several different types of venom components can have deleterious effects on endothelial cells. Furthermore, any proposed mechanism of action of hemorrhagic toxins must take into account the extremely rapid action observed *in vivo*, which takes place within the range of a few minutes (OWNBY *et al.*, 1990; LOMONTE *et al.*, 1994).

The results obtained with myotoxin II demonstrate that a direct cytotoxic action on endothelial cells does not necessarily lead to hemorrhage *in vivo*, since it is known that this toxin does not cause bleeding (LOMONTE and GUTIERREZ, 1989; LOMONTE *et al.*, 1993, 1994). In addition, the observed cytotoxic activity of myotoxin II on endothelial cells is of interest, as it provides a possible explanation for its ability to rapidly increase vascular permeability *in vivo* (LOMONTE and GUTIERREZ, 1989; LOMONTE *et al.*, 1993, 1994), a biological action also observed with other lysine-49 phospholipases A_2 of snake venom origin (LIU *et al.*, 1991). The cytotoxic action of myotoxin II on endothelial cells, in comparison to cultured skeletal muscle myoblasts, is currently being characterized.

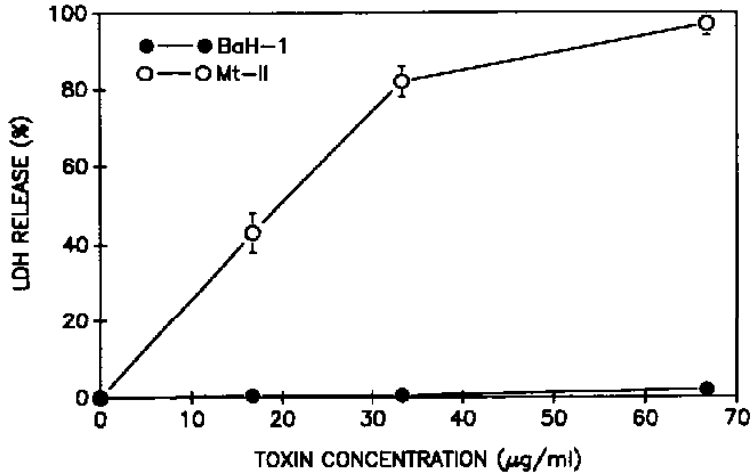

FIG. 1. PURIFIED HEMORRHAGIC TOXIN BaH-1 IS NOT DIRECTLY CYTOTOXIC TO MURINE CAPILLARY ENDOTHELIAL CELLS IN CULTURE.

The indicated concentrations of toxin were incubated with cells for 3 hr at 37°C, in a volume of 150 μl/well, as described in Materials and Methods. Then, supernatants were assayed for lactic dehydrogenase (LDH) activity. Results are expressed as percentage, considering as 0% and 100% the activity of supernatants of cultures treated with plain medium or medium containing 0.1% Triton X-100, respectively. Each point represents the mean ± S.D. of triplicate wells. Myotoxin II (Mt-II), a non-hemorrhagic component purified from the same venom, is included for comparison.

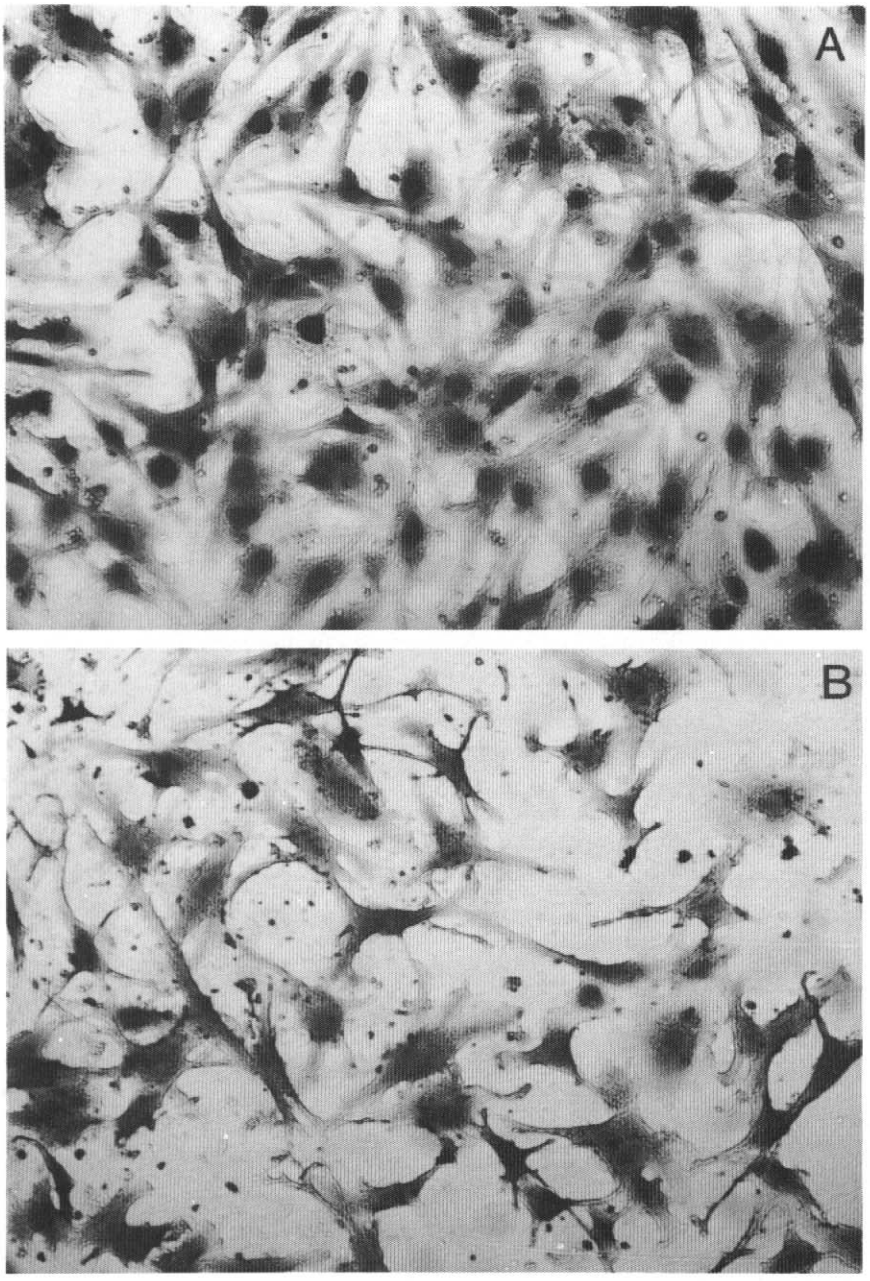

FIG. 2. MORPHOLOGY OF MURINE CAPILLARY ENDOTHELIAL CELLS TREATED WITH HEMORRHAGIC TOXIN BaH-1.

A: Control culture; B: cells after 3 hr of incubation at 37°C with toxin BaH-1 (32 μg/ml). Areas of cell detachment can be seen together with apparently normal cells. Hematoxylin-eosin stain, magnification $\times 32$.

In conclusion, the present results suggest that the hemorrhagic action of BaH-1 is not related to a direct cytotoxic effect on capillary endothelium. Since endothelial cells are evidently affected *in vivo* by hemorrhagic toxins, this effect should be an indirect consequence of toxin action, perhaps initiated by the proteolytic degradation of basal lamina constituents.

Note added in proof—A recent article by OBRIG *et al.* (1993, *Microvasc. Res.* **46,** 412–416) reported direct cytotoxic effects of hemorrhagic toxins from *Crotalus atrox* and *C. ruber ruber* on human endothelial cells *in vitro*. Their conclusion is based on the decreased neutral red uptake by cells, after 24 hr (for HT-1 and HT-2 of *C. ruber ruber*) or 72 hr (for HT-a and HT-d of *C. atrox*) of exposure to the toxins. Since the method utilized required two washing steps of the cultures, it is not clear if the resulting decreased color signal reflects a true cytotoxic effect, as stated by the authors, or a loss of cells due to proteolytic detachment from their substratum. Unfortunately, no attempt was made to describe morphological changes, including detachment, in the cultures treated with the hemorrhagic toxins. Our present report shows that detachment is an evident phenomenon in the case of BaH-1 of *B. asper*, implying that the evaluation of direct cytotoxic effects of hemorrhagic toxins should be performed by techniques that avoid washing steps, such as by measuring the release of intracellular markers, or other signs of rapid cell damage, that would be compatible with the *in vivo* observation of capillary disruption within the range of minutes.

Acknowledgements—We thank Dr. A. MANTOVANI for the valuable gift of tEnd cells, and ING-MARIE NILSSON, MARGARETA VERDRENGH, and BRITTA PALMER for excellent technical support. This study was supported by AID (Grant DHR-5544-6-00-1064-00), the Swedish Agency for Research Cooperation with Developing Countries (SAREC), and the Costa Rican National Council for Science and Technology (CONICIT).

REFERENCES

ARAKI, S., ISHIDA T., YAMAMOTO, T., KAJI, K. and HAYASHI, H. (1993) Induction of apoptosis by hemorrhagic snake venom in vascular endothelial cells. *Biochem. Biophys. Res. Commun.* **190,** 148–153.

BARAMOVA, E. N., SHANNON, J. D., BJARNASON, J. B. and FOX, J. W. (1989) Degradation of extracellular matrix proteins by hemorrhagic metalloproteinases. *Archs Biochem. Biophys.* **275,** 63–71.

BJARNASON, J. B. and FOX, J. W. (1988–1989) Hemorrhagic toxins from snake venoms. *J. Toxicol.-Toxin Rev.* **7,** 121–209.

BJARNASON, J. B., HAMILTON, D. and FOX, J. W. (1988) Studies on the mechanism of hemorrhage production by five proteolytic hemorrhagic toxins from *Crotalus atrox* venom. *Biol. Chem. Hoppe-Seyler* **369,** 121–129.

BORKOW, G., GUTIERREZ, J. M. and OVADIA, M. (1993) Isolation and characterization of synergistic hemorrhagins from the venom of the snake *Bothrops asper*. *Toxicon* **31,** 1137–1150.

BUSSOLINO, F., DE ROSSI, M., SICA, A., COLOTTA, F., WANG, J. M., BOCCHIETTO, E., PADURA, I. M., BOSIA, A., DEJANA, E. and MANTOVANI, A. (1991) Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. *J. Immunol.* **147,** 2122–2129.

FRANCIS B., GUTIERREZ, J. M., LOMONTE, B. and KAISER, I. I. (1991) Myotoxin II from *Bothrops asper* (Terciopelo) venom is a lysine-49 phospholipase A_2. *Archs Biochem. Biophys.* **284,** 352–359.

GUTIERREZ, J. M. and LOMONTE, B. (1989) Local tissue damage induced by *Bothrops* snake venoms: a review. *Mem. Inst. Butantan* **51,** 211–223.

HITE, L. A., FOX, J. W. and BJARNASON, J. B. (1992) A new family of proteinases is defined by several snake venom metalloproteinases. *Biol. Chem. Hoppe-Seyler* **373,** 381–385.

KONDO, H., KONDO, S., IKEZAWA, H., MURATA, R. and OHSAKA, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom. *Jpn. J. Med. Sci. Biol.* **13,** 43–51.

LIU, C. S., CHEN, J. M., CHANG, C. H., CHEN, S. W., TENG, C. M. and TSAI, I. H. (1991) The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochim. Biophys. Acta* **1077,** 362–370.

LOMONTE, B. and GUTIERREZ, M. J. (1989) A new muscle damaging toxin, myotoxin II, from the venom of snake *Bothrops asper* (terciopelo). *Toxicon* **27,** 725–733.

LOMONTE, B., TARKOWSKI, A. and HANSON, L. Å. (1993) Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* **17,** 93–105.

LOMONTE, B., LUNDGREN, J., JOHANSSON, B. and BAGGE U. (1994) The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. *Toxicon.*

MARUYAMA, M., SUGIKI, M., YOSHIDA, E., SHIMAYA, K. and MIHARA, H. (1992) Broad substrate specificity of snake venom fibrinolytic enzymes: possible role in haemorrhage. *Toxicon* **30,** 1387–1397.

MOREIRA, L., GUTIERREZ, J. M., BORKOW, G. and OVADIA, M. (1992) Ultrastructural alterations in mouse capillary blood vessels after experimental injection of venom from the snake *Bothrops asper* (terciopelo). *Exp. Mol. Path.* **57,** 124–133.

OWNBY, C. L. (1990) Locally acting agents: myotoxins, hemorrhagic toxins and dermonecrotic factors. In: *Handbook of Toxinology*, pp. 602–654 (SHEIR, W. T. and MEBS, D., Eds). New York: Marcel Dekker.

OWNBY, C. L., BJARNASON, J. W. and TU, A. T. (1978) Hemorrhagic toxins from rattlesnake (*Crotalus atrox*) venom. Pathogenesis of hemorrhage induced by three purified toxins. *Am. J. Path.* **93,** 201–218.

OWNBY, C. L. and GEREN, C. R. (1987) Pathogenesis of hemorrhage induced by hemorrhagic proteinase IV from timber rattlesnake (*Crotalus horridus horridus*) venom. *Toxicon* **25,** 517–526.

OWNBY, C. L., NIKA, T., IMAI, K. and SUGIHARA, H. (1990) Pathogenesis of hemorrhage induced by bilitoxin, a hemorrhagic toxin isolated from the venom of the common cantil (*Agkistrodon bilineatus bilineatus*). *Toxicon* **28,** 837–846.

PAINE M. J., DESMOND, H. P., THEAKSTON, R. D. and CRAMPTON, J. M. (1992) Purification, cloning, and molecular characterization of a high molecular weight hemorrhagic metalloprotease, jararhagin, from *Bothrops jararaca* venom. *J. biol. Chem.* **267,** 22,869–22,876.

TAKACS, Z., RETZIOS, A. D. and MARKLAND, F. S. (1992) A hemorrhagic (basement membrane degrading) zinc metalloproteinase from Southern copperhead venom. *Matrix* Suppl. 1, 101–103.

VI

Pergamon

Biochemical Pharmacology, Vol. 47, No. 9, pp. 1509–1518, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0004-5

NEUTRALIZATION OF THE CYTOLYTIC AND MYOTOXIC ACTIVITIES OF PHOSPHOLIPASES A_2 FROM *BOTHROPS ASPER* SNAKE VENOM BY GLYCOSAMINOGLYCANS OF THE HEPARIN/HEPARAN SULFATE FAMILY

BRUNO LOMONTE,*†‡ ANDREJ TARKOWSKI,*§ ULF BAGGE‖ and LARS Å. HANSON*

Departments of *Clinical Immunology, §Rheumatology and ‖Anatomy, University of Göteborg, Göteborg, Sweden; and ‡Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

(*Received* 23 *September* 1993; *accepted* 24 *December* 1993)

Abstract—Basic phospholipases A_2 from the venom of *Bothrops asper* exhibit skeletal muscle damaging activity *in vivo*, and cytolytic activity to a variety of cell types in culture. Glycosaminoglycans of the heparin/heparan sulfate family were found to be potent blockers of the cytolytic action *in vitro*, and, as well, to be able to neutralize the muscle damaging activity of purified myotoxins and crude venom *in vivo*. However, the neutralizing effect of heparins was more potent *in vitro* than *in vivo*. The cytolytic activity of myotoxin II (a lysine-49 phospholipase A_2 isoform) and its inhibition by heparin was characterized. The neutralizing effect of heparin did not depend on its anticoagulant activity, since both standard heparin and heparin with low affinity for antithrombin (LA-heparin) had a similar efficiency. Heparan sulfate and low molecular mass heparin (5 kDa) also neutralized myotoxin II. In contrast, different heparin-derived disaccharides were unable to block cytolysis, implying a requirement for a longer carbohydrate chain structure for the interaction with the protein. By affinity chromatography and gel diffusion, it was demonstrated that heparins form a complex with all isoforms of basic venom myotoxins, held at least in part by electrostatic interactions. The phospholipase A_2 activity of myotoxin III, a related aspartate-49 isoform from the same venom, was unaffected by heparins, despite the fact that its myotoxic activity was inhibited, indicating a dissociation of the two actions.

Key words: heparin; phospholipase A_2; cytotoxicity; myotoxin; snake venom

In recent years, heparin has been shown to interact *in vitro* with several types of PLA_2s¶, (EC 3.1.1.4) [1–3] and in some cases, to inhibit their enzymatic activity. PLA_2s are notoriously abundant and widely distributed in snake venoms, in which they have acquired a variety of toxic activities in the course of evolution, such as neurotoxicity, myotoxicity, anticoagulant effect, and edema-forming activity [4, 5].

Non-immunologic inhibitors of myotoxic PLA_2s could be of interest therapeutically, especially considering that antivenom immunoglobulins have a limited efficacy in preventing the muscle damage that follows envenomation [6, 7]. In addition, inhibitors may constitute useful tools for understanding the mechanism of action of myotoxic PLA_2s. The neutralization of myotoxic activity of the venom of *Bothrops jararacussu*, a crotalid species from Brazil, by heparin has been reported [8, 9]. This interesting finding is further investigated in the present work, using myotoxin II, a basic PLA_2 purified from the venom of *B. asper* [10], and with special interest on heparin derivatives with little or no effect on the coagulation system, i.e. heparin with low affinity for antithrombin. Myotoxin II is a natural PLA_2 isoform devoid of enzymatic activity, mainly due to the critical amino acid substitution at position 49 (Asp→Lys) [11], but still displays myotoxic activity [10]. In some experiments, an isoform that has PLA_2 activity, myotoxin III [12], was utilized to investigate the effect of heparins on the enzymatic activity of these proteins. In order to have a well standardized system for the characterization of the neutralizing ability of heparins, an *in vitro* model of cytotoxicity was developed, using two cell types as targets, skeletal muscle myoblasts and capillary endothelial cells.

MATERIALS AND METHODS

Venoms and myotoxic PLA_2s from B. asper. Crude venom of *B. asper* specimens from Costa Rica (Pacific region) kept at the serpentarium of the Instituto Clodomiro Picado (University of Costa Rica) was fractionated by ion-exchange chromatography on CM-Sephadex C-25 as previously described [10] to obtain pure myotoxin II, as evaluated by SDS–PAGE [13] and cathodic native PAGE at pH 4.3 [14]. *B. asper* myotoxin III [12] was kindly provided by Dr J.M. Gutiérrez, University of Costa Rica. Crude venoms of *Agkistrodon piscivorus piscivorus, Bothrops jararacussu, Crotalus durissus terrificus, C. viridis viridis, Naja naja atra,*

† Corresponding author: B. Lomonte, Department of Clinical Immunology, University of Göteborg, Guldhedsgatan 10, S-413 46 Göteborg, Sweden. FAX 46-31-826791.

¶ Abbreviations: PLA_2, phospholipase A_2; LA-heparin, heparin with low affinity for antithrombin; CK, creatine kinase; LDH, lactic dehydrogenase; PAGE, polyacrylamide gel electrophoresis.

Trimeresurus flavoviridis, and *Vipera berus*, were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.).

Glycosaminoglycans. Standard heparin (5000 IE/mL), heparin with low affinity for antithrombin (LA-heparin; 7 IU anti-factor Xa/mg, M_r 15,000), and low molecular mass heparin (Fragmin®, 25,000 IE/mL, M_r 5000) were provided by Kabi Pharmacia (Sweden), through the courtesy of Dr L.O. Andersson. Heparan sulfate (from bovine intestinal mucosa, H-7641) and a series of heparin-derived disaccharides were purchased from Sigma. A purified chondroitin sulfate preparation (from bovine cartilage) was kindly provided by M. Maccarana (Uppsala University, Sweden).

Myotoxic activity. Myotoxicity was evaluated in mice (Swiss, 20–24 g) by injecting either crude *B. asper* venom or pure myotoxins, alone or after incubation with varying amounts of heparins (15 min at room temperature), by the i.m. route (gastrocnemius), and then measuring the serum CK (EC 2.7.3.2) activity (kit No. 520, Sigma) after 3 hr [6]. To confirm data from the enzymatic assay, samples of injected tissue were evaluated histologically on hematoxylin-eosin stained sections. In addition, neutralization of *B. asper* venom myotoxicity by heparins was evaluated qualitatively using an intravital microscopic technique to study the effect of local application on the mouse cremaster muscle, as previously described [15].

The ability of LA-heparin to neutralize the myotoxic activity of several snake venoms, in addition to that of *B. asper*, was investigated similarly, using the serum CK levels after 3 hr of injection as an indicator of muscle damage. The ratio of LA-heparin/venom utilized in these screenings was 5 μg/μg.

In vitro *cytotoxicity*. The activity of myotoxin II was quantified *in vitro* by a cytotoxicity assay using two cell lines. L6 rat myoblasts (ATCC CRL 1458) were kindly provided by Dr M. Thelestam (Karolinska Institute, Sweden), whereas tEnd cells, a polyoma virus-transformed mouse endothelial cell line of capillary origin [16] were a kind gift from Dr A. Mantovani (Istituto di Ricerche Farmacologiche Mario Negri, Italy). Cells were routinely grown in Iscove's medium (Gibco, Paisley, U.K.) supplemented with 10% fetal calf serum (FCS; Biological Ind., Haemek, Israel), 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 0.05 mg/mL gentamycin. In order to quantify cytotoxicity, cells were seeded at 1–4 $\times$ 10^4/well in 96-well plates and grown for 2–4 days until almost confluent. At the moment of the assay, culture medium was removed and replaced with 150 μL/well of medium with 1% FCS containing different amounts of myotoxin II. The FCS concentration was lowered to 1% in order to minimize the basal LDH (EC 1.1.1.27) activity of the medium. After 3 hr of incubation at 37° (selected after time-course experiments), 100 μL of supernatant were assayed for LDH released from damaged cells (kit No. 500, Sigma). As reference values for 100 and 0% cytotoxicity, cells were incubated with 0.1% Triton X-100-containing medium, or plain medium, respectively. All samples were assayed in triplicate wells.

For neutralization experiments, myotoxin II (at concentrations that would induce 100% cytotoxicity if uninhibited, i.e. 10 or 20 μg/150 μL/well for tEnd and L6 cells, respectively) was preincubated with the different tested agents for 15 min at room temperature, and then applied to cultures as described above.

In order to determine if the inhibitory activity of heparins on myotoxin-induced cytolysis was due to an effect on the target cells, cultures were preincubated with heparin (170 μg/mL) for 2 hr at 37°, washed, and then exposed to myotoxin II as described above.

A neutralizing monoclonal antibody to *B. asper* myotoxins, MAb-3 [17] was also tested in this cytotoxicity assay, to investigate if a similar neutralizing potency would be obtained in comparison to that observed *in vivo* [18]. MAb-3 was obtained from ascitic fluid in BALB/c mice, and partially purified by ammonium sulfate precipitation. Its final concentration was estimated by radial immunodiffusion [19] using a mouse IgG_1 standard (Sigma).

Edema-forming activity. The ability of LA-heparin to inhibit the edema induced by myotoxin II was tested in the footpads of mice, by injecting 100 μg of the toxin, either alone or after incubation with LA-heparin (5 μg/μg toxin), in 50 μL of PBS by s.c. route. Edema was quantified at different time points by measuring the increase in footpad thickness with a low-pressure spring caliper [20]. Control mice received an injection of 50 μL of PBS.

Phospholipase A_2 assay. An indirect hemolytic assay in suspension, based on the gel diffusion technique described by Gutiérrez *et al.* [21] was utilized. Washed sheep red blood cells were suspended at 1.5% (v/v) in 0.12 M NaCl, 0.04 M sodium phosphate buffer, pH 8.1, containing 1% egg yolk as a source of phospholipids and 0.09 mM $CaCl_2$. To 500 μL of this preparation, 5 μL (containing 5 μg) of myotoxin III alone, or after preincubation with heparins (at a ratio of 5 μg heparin/μg toxin), were added and incubated for 30 min at 37°. Then, 3 mL of buffer were added to each tube, and hemolysis was read at 540 nm after centrifugation. To assure the PLA_2 dependency of the lysis and the lack of direct hemolysis, parallel myotoxin samples were run using red blood cells in the absence of egg yolk phospholipids.

Affinity chromatography. Crude *B. asper* venom (25 mg) was applied to a column of heparin-agarose (Sigma) equilibrated with PBS, pH 7.2. After the absorbance at 280 nm of the eluent returned to baseline, elution of the heparin-binding fraction was performed by either a stepwise or a linear gradient to 1 M NaCl. The eluted fraction was analysed by SDS–PAGE and native cathodic PAGE as described above.

Gel diffusion. The interaction between heparins and crude venom or myotoxin II was tested by gel diffusion in 1% agarose-PBS plates [22]. After 24 hr of incubation at room temperature, gels were washed with PBS and the precipitates stained with Coomassie blue R-250.

Cell surface heparan sulfate treatments. The possible role of cell surface heparan sulfate in the

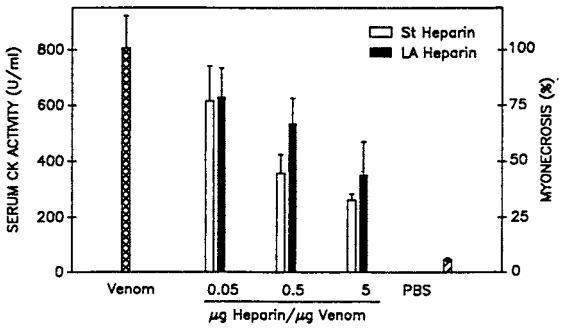

Fig. 1. Heparins neutralize the myotoxic action of whole *B. asper* venom in mice. Venom and different amounts of heparins were mixed, incubated 15 min at room temperature, and then injected i.m. into groups of four mice (50 μg venom/mouse). After 3 hr, CK levels in serum were determined as an indicator of skeletal muscle damage (% myonecrosis). CK values obtained with venom alone were taken as 100%. St heparin: standard heparin (empty bars); LA-heparin: low affinity heparin (filled bars); PBS: phosphate-buffered saline. Bars represent means ± SD of four determinations. All values are significantly ($P < 0.05$) lower than the venom control.

cytotoxic mechanism of myotoxin II was investigated by (a) heparitinase digestion of cell cultures, (b) inhibition of heparan sulfate sulfation with sodium chlorate, and (c) the use of a cell mutant with a defect in heparan sulfate biosynthesis.

For heparitinase treatment, cell cultures were incubated with 1.25 U/well of heparinase III (Sigma), in 100 μL of PBS, for 2 hr at 37° [23]. Control cells were treated similarly but omitting the enzyme. Then, wells were washed twice with culture medium, and the cytotoxicity induced by myotoxin II was determined as described above. For chlorate treatment, cells were plated in microwells with medium containing 10 mM sodium chlorate [24, 25] and grown for 2 days. At the moment of the assay, myotoxin II was added in chlorate-containing medium, and cytotoxicity was determined. Chlorate was omitted in control cultures. Finally, the cytotoxic action of myotoxin II was also quantified on two CHO cell lines, one defective in the synthesis of heparan sulfate (CHO-*pgs*D-677), and the wild type control (CHO-K1) [26], kindly provided by Dr J.D. Esko (University of Alabama-Birmingham, U.S.A.).

RESULTS

When whole *B. asper* venom was preincubated with either standard or LA-heparin, and subsequently injected into mice, its myotoxic action was significantly reduced in a dose-dependent manner (Fig. 1). This result was confirmed by histological evaluation and by the use of intravital microscopy. In the latter system, widespread muscle fiber damage regularly developed 4–6 min after application of venom alone, whereas, when it was mixed with standard or LA-heparin before application, muscle fibers were protected throughout the observation period of 30 min. In contrast to LA-heparin, standard heparin markedly increased the hemorrhage induced by the venom, evident both histologically and intravitally.

The ability of LA-heparin to neutralize the muscle-damaging activity of venoms from other species was explored. It was found that the venoms of *A. p. piscivorus*, *B. jararacussu*, and *T. flavoviridis*, were also significantly neutralized regarding myotoxic activity, as judged by the reduction in the serum CK levels measured at 3 hr. However, not all venoms tested were susceptible to heparin neutralization of myotoxic activity (Table 1).

To analyse in more detail the neutralizing ability of heparins towards myotoxins of *B. asper* venom, an *in vitro* cytotoxicity assay was developed using purified myotoxin II. This toxin was cytolytic not only to L6 myoblasts, but also to tEnd cells, in the concentration range of 25–150 μg/mL. Unexpectedly, endothelial cells were significantly more susceptible than myoblasts to the cytotoxic action of

Table 1. Effect of LA-heparin on the myotoxic activity of different snake venoms, tested by preincubation and subsequent *in vivo* administration to mice*

Species	Dose (μg)	Serum CK levels at 3 hr (U/mL)		P value†
		Venom alone	Venom + LA-heparin	
Agkistrodon p. piscivorus	50	768 ± 101	354 ± 72	<0.01
Bothrops jararacussu	50	370 ± 60	181 ± 59	<0.01
Crotalus durissus terrificus	5	298 ± 109	382 ± 80	>0.1
Crotalus viridis viridis	50	617 ± 118	484 ± 172	>0.1
Naja naja atra	5	454 ± 326	418 ± 180	>0.1
Trimeresurus flavoviridis	50	1086 ± 220	393 ± 69	<0.01
Vipera berus	50	399 ± 66	408 ± 111	>0.1

* The ratio of LA-heparin/venom in the preincubation mixture was 5 μg/μg. The dose utilized the case of *C. d. terrificus* and *N. a. atra* venoms was 5 μg/mouse (instead of 50 μg/mouse) due to their highly lethal neurotoxic activity.

† Serum CK values are presented as means ± SD, using four animals in each group. Normal CK levels in mice injected with saline were 51 ± 11 U/mL. The means between each pair (venom alone and venom + LA-heparin) were compared by two-tailed Student's *t*-test.

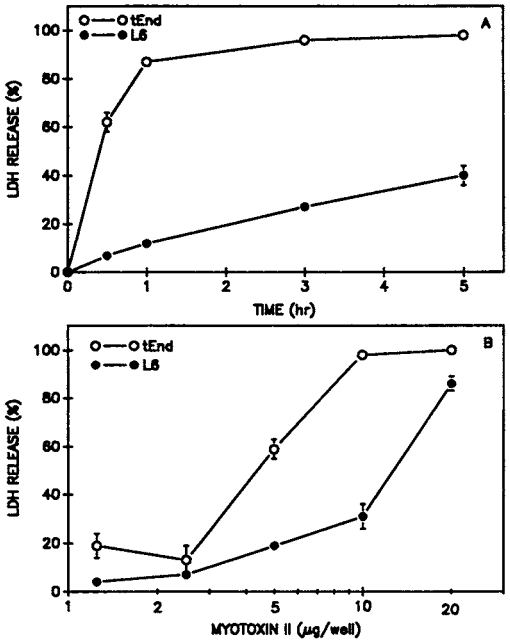

Fig. 2. Myotoxin II is cytotoxic to cultured rat myoblasts (L6) and mouse capillary endothelial cells (tEnd). (A) Time-course of LDH release from damaged tEnd (○) and L6 (●) cells after incubation with myotoxin II (10 μg/well; see Materials and Methods). LDH release is expressed as a percentage, considering the enzyme activity of Triton X-100 treated culture supernatants as 100%. (B) Dose–response curves of cytotoxic activity of myotoxin II on tEnd (○) and L6 (●) cells, measured by release of LDH at 3 hr. Each point represents mean ± SD of triplicate wells.

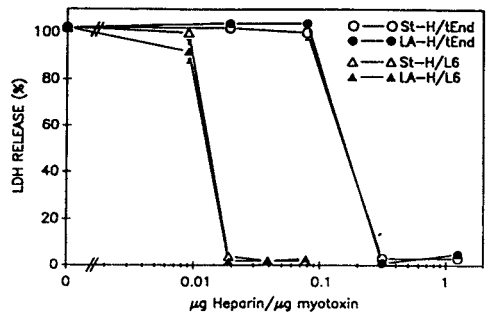

Fig. 3. Standard heparin and LA-heparin block the cytotoxic activity of myotoxin II. Either standard (St-H; empty symbols) or low affinity (LA-H; filled symbols) heparins were mixed with myotoxin II at the indicated proportions, incubated for 15 min at room temperature, and then assayed for cytotoxic activity on tEnd (circles) and L6 (triangles) cells. The toxin challenge for tEnd and L6 cells was 10 and 20 μg/well, respectively. Cytotoxicity was measured by the release of LDH from cells at 3 hr. Each point represents mean ± SD of triplicate wells.

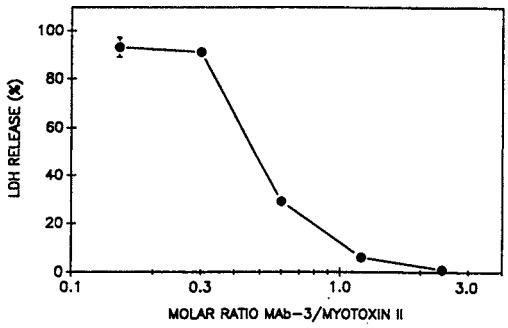

Fig. 4. Neutralization of the cytotoxic activity of myotoxin II on tEnd endothelial cells by monoclonal antibody MAb-3. MAb-3 and myotoxin II mixed at different molar ratios were preincubated for 15 min at room temperature, and then assayed for cytotoxic activity on tEnd cells. Cytotoxicity is estimated by the release of LDH, under the same conditions as in Fig. 3. Each point represents mean ± SD of triplicate wells.

myotoxin II (Fig. 2A and B). Morphologically, both cell types showed, after exposure to the toxin, an abundant cytoplasmic granulation, followed by an apparent dissolution of the membrane, without detachment, similar to the appearance of detergent-treated cultures. An incubation time of 3 hr (Fig. 2A) and a toxin challenge of 20 and 10 μg/well, for L6 and tEnd cells, respectively (Fig. 2B), were selected for all subsequent experiments, to assure 100% cytotoxicity in the absence of a neutralization effect. When preincubated with myotoxin II, standard as well as LA-heparin blocked its cytolytic activity on both cell types. The neutralizing potency of heparins in this assay system was considerably high, since cells were completely protected at approximate ratios of 0.02 and 0.3 μg heparin/μg myotoxin II, for L6 and tEnd cells, respectively, with no observable differences in the inhibitory efficiency of the two heparin types (Fig. 3). Since the average molecular mass of both heparins utilized (15 kDa) is similar to that of myotoxin II, the heparin/toxin ratios expressed as μg/μg roughly correspond to molar ratios. Under identical conditions, the myotoxicity-neutralizing antibody MAb-3 was also able to inhibit the action of myotoxin II in the cell culture system, completely preventing its effect at an approximate molar ratio of 1:1 (Fig. 4).

A complete neutralization of cytolysis caused by myotoxin II was also achieved with heparan sulfate and the low molecular weight heparin, although not with a variety of heparin-derived disaccharides or with chondroitin sulfate (Table 2). On the other hand, pretreatment of the cells with heparins, followed by washing, had no protective effect on myotoxin II-induced cytolysis (data not shown).

Since myotoxin II induces edema *in vivo*, and was found to be directly cytotoxic to endothelial cells in culture, the ability of heparin to inhibit the edema-forming activity of the toxin was investigated. A significant inhibition of edema in the mouse footpad assay was achieved by preincubation of myotoxin II with LA-heparin (Fig. 5).

Table 2. Heparan sulfate and low molecular mass heparin (Fragmin®), but not heparin disaccharides or chondroitin sulfate, neutralize the cytotoxic activity of myotoxin II on L6 myoblasts and tEnd endothelial cells

Tested agent, ratio	%LDH release, mean ± SD L6 cells	tEnd cells
Myotoxin II control*	96 ± 3	99 ± 2
Heparan sulfate, 0.0075 μg/μg myotoxin	NT	101 ± 1
Heparan sulfate, 0.075 μg/μg myotoxin	95 ± 1	82 ± 2
Heparan sulfate, 0.75 μg/μg myotoxin	4 ± 1	2 ± 1
Chondroitin sulfate, 1 μg/μg myotoxin	NT	96 ± 3
Chondroitin sulfate, 10 μg/μg myotoxin	NT	99 ± 7
Fragmin®, 0.75 μg/μg myotoxin	3 ± 3	5 ± 1
Heparin disaccharides, 0.75 μg/μg myotoxin		
I-A: α-ΔUA-2S-[1→4]-GlcNAc-6S	97 ± 3	NT
II-A: α-ΔUA-[1→4]-GlcNAc-6S	99 ± 2	NT
III-A: α-ΔUA-2S-[1→4]-GlcNAc	101 ± 2	NT
I-H: α-ΔUA-2S-[1→4]-GlcN-6S	102 ± 1	NT
II-H: α-ΔUA-[1→4]-GlcN-6S	102 ± 1	NT
I-S: α-ΔUA-2S-[1→4]-GlcNS-6S	101 ± 1	NT
II-S: α-ΔUA-[1→4]-GlcNS-6S	99 ± 1	NT
III-S: α-ΔUA-2S-[1→4]-GlcNS	100 ± 2	NT
IV-S: α-ΔUA-[1→4]-GlcNS	100 ± 2	NT

NT: not tested.
* The toxin challenge was 10 and 20 μg/well for tEnd and L6 cells, respectively.

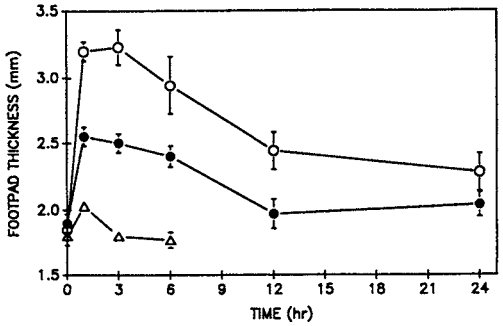

Fig. 5. Neutralization of the edema induced by myotoxin II in the mouse footpad by LA-heparin. Myotoxin II was mixed with either saline (○) or LA-heparin (●) at a ratio of 5 μg heparin/μg myotoxin, and incubated for 15 min at room temperature. Then, 50 μL of each solution (containing 100 μg myotoxin II) were injected s.c. into the footpads of mice. Control mice received 50 μL of saline only (△). Edema was estimated by measuring the footpad thickness at the indicated time points, as described in Materials and Methods. Each point represents the mean ± SD of four animals.

The heparin-binding components in *B. asper* venom were isolated by affinity chromatography, eluting at approximately 0.5 M NaCl concentration. SDS–PAGE of this fraction showed a main band of 14–15 kDa under reducing conditions, which corresponds to the subunit molecular mass of myotoxins, and very small amounts of few other components (Fig. 6A). Native PAGE for basic proteins showed that all described myotoxin isoforms bound to the immobilized heparin column (Fig. 6B). Gel diffusion demonstrated that heparins form a precipitable complex with venom myotoxins (Fig. 6C).

Since affinity chromatography and electrophoresis showed that heparin interacts not only with myotoxin II, but also with other isoforms present in this venom, the effect of heparins on the enzymatic and myotoxic activities of myotoxin III was investigated. Both types of heparins significantly reduced the myotoxic effect of this isoform *in vivo*, without inhibiting its enzymatic activity in an indirect hemolysis assay (Fig. 7). Control erythrocyte suspensions treated with myotoxin III showed no hemolysis in the absence of phospholipids.

Since free heparan sulfate was found to block the cytolytic action of myotoxin II (Table 2), the possible role of cell surface heparan sulfate in the mechanism of cytotoxicity was investigated (Fig. 8). Both heparitinase and chlorate treated L6 and tEnd cells were equally susceptible to myotoxin II, in comparison with their respective controls (Fig. 8A and 8B). In agreement with this, myotoxin II induced a comparable cytolytic effect in heparan sulfate defective mutant cells CHO-*pgs*D-677 and CHO-K1 control cells (Fig. 8C).

DISCUSSION

This work demonstrates that glycosaminoglycans of the heparin/heparan sulfate family block the cytolytic action of basic myotoxic PLA_2s from the venom of *B. asper*, both in cell culture and *in vivo*. This inhibition is clearly due to the formation of a complex, which is held, at least in part, by electrostatic interactions between the negatively-charged groups of heparins and the numerous positively-charged amino acid residues of myotoxins, which are highly basic proteins. This is supported

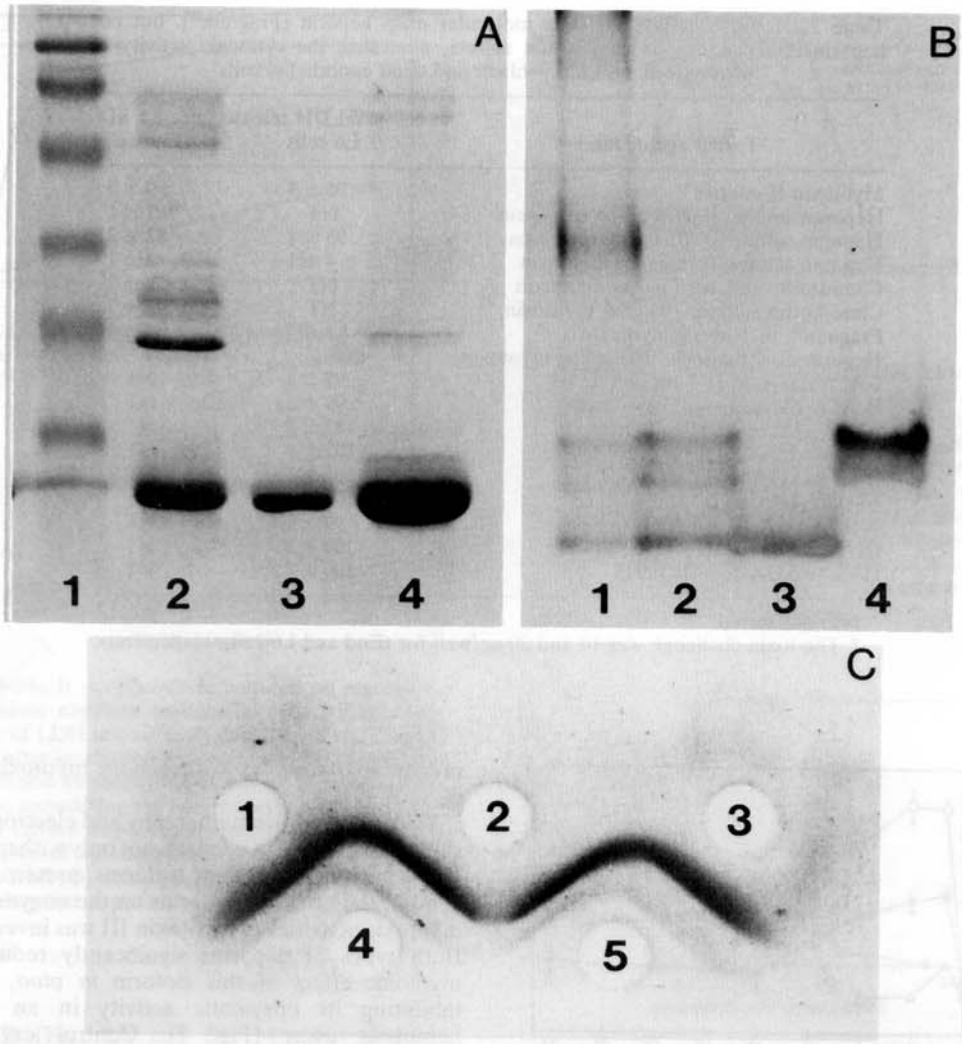

Fig. 6. *B. asper* venom myotoxins bind to heparin. Whole venom was fractionated on a column of heparin-agarose (Sigma) as described in Materials and Methods, and a heparin-binding fraction was obtained by salt elution. (A) Analysis of the heparin-binding fraction by SDS–PAGE, 15% (anode at the bottom, Coomassie blue R-250 stain). Lane 1: molecular mass standards (106, 80, 49.5, 32.5, 27.5 and 18.5 kDa); lane 2: crude *B. asper* venom, 10 μg; lane 3: myotoxin II, 5 μg; lane 4: heparin-binding fraction, 20 μg. All samples were reduced with 2-mercaptoethanol at 95°. (B) Analysis of the heparin-binding fraction by cathodic PAGE under native conditions (Coomassie R-250 stain, cathode at the bottom). Lane 1: crude *B. asper* venom, 10 μg; lane 2: heparin-binding fraction, 20 μg; lane 3: myotoxin II, 10 μg; lane 4: mixture of myotoxins I and III, 10 μg. (C) Gel diffusion in 1% agarose–PBS. Wells were filled with 20 μL of the following solutions: 1 and 3: crude *B. asper* venom, 20 mg/mL; 2: myotoxin II, 0.5 mg/mL; 4: standard heparin, 0.17 mg/mL; 5: LA-affinity heparin, 0.17 mg/mL. Coomassie blue R-250 stain.

by the direct observation of a precipitate in gel diffusion, and by the dissociation of myotoxins from the heparin affinity column with an increasing ionic strength gradient. Melo *et al.* [9], using gel filtration, also obtained evidence for complex formation between heparin and a myotoxic PLA_2 from *B. jararacussu* venom, that has similar antigenic [27] and physicochemical [28] characteristics to *B. asper* myotoxins. Venoms from many species of *Bothrops*, distributed in Latin America, as well as other crotalids such as *Trimeresurus flavoviridis* from Japan, contain components that cross-react antigenically with *B. asper* myotoxins [27], and therefore are presumably myotoxic PLA_2s with similar properties, as confirmed in several cases [29–31]. Thus, the present results might be relevant also for other myotoxic PLA_2s. Indeed, a significant reduction in the muscle-damaging activity of crude venoms from crotalid species other than *B. asper* was obtained by the use of LA-heparin. Interestingly, the venoms that were inhibited correspond to those containing PLA_2s antigenically-related to those of *B. asper*, *T. flavoviridis* [27] and *B. jararacussu* [27], the latter result confirming the original report by

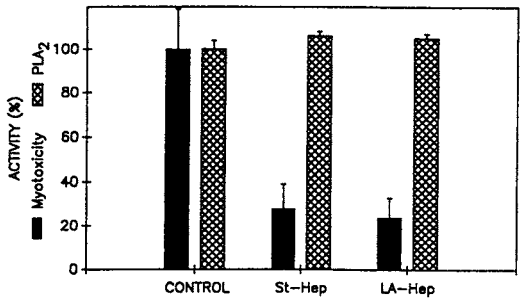

Fig. 7. Heparins block the myotoxic activity of myotoxin III, but not its PLA_2 activity. Either standard (St-Hep) or low affinity (LA-Hep) heparins were mixed with myotoxin III at a ratio of 5 μg heparin/μg myotoxin, and incubated for 15 min at room temperature. Then, mixtures were assayed for *in vivo* myotoxic activity (50 μg myotoxin/mouse) or *in vitro* PLA_2 activity (5 μg myotoxin/tube). Activities are expressed as a percentage, considering the activity of the toxin alone as 100%. Bars represent means ± SD of triplicate determinations.

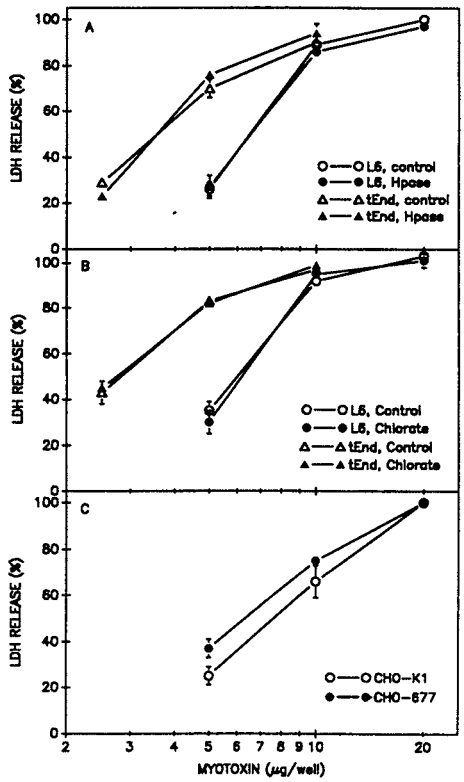

Fig. 8. Cell surface heparan sulfate is not required for the cytolytic activity of myotoxin II. (A) Cell cultures of myoblasts (L6; circles) or endothelial cells (tEnd; triangles) were treated with heparitinase (1.25 U/100 μL PBS/well; Hpase) for 2 hr at 37° (filled symbols) or PBS alone as a control (empty symbols), washed, and then exposed to different amounts of myotoxin II. Cytotoxicity is estimated by the release of LDH, under the same conditions as in Fig. 3. All points represent means ± SD of triplicate wells. No significant differences are found between the two treatments. (B) Cell cultures of myoblasts (L6; circles) or endothelial cells (tEnd; triangles) were grown for 2 days in the presence of 10 mM sodium chlorate-containing medium (filled symbols) or normal medium as a control (empty symbols), and then exposed to myotoxin II. No significant differences are found between the two treatments. (C) Chinese hamster ovary cells were used as targets for myotoxin II action. CHO-*pgs*D-677 (●) is a mutant cell line defective in heparan sulfate synthesis and CHO-K1 (○) is the wild type control. LDH was determined at 3 hr. Only the difference between the values obtained with 5 μg toxin is statistically significant ($P < 0.05$).

Melo *et al.* [8]. Similarly, although the venom of *A. p. piscivorus* has not been tested for the presence of PLA_2s antigenically-related to those of *B. asper*, the closely related species *A. bilineatus* contains such component(s) [32]. Also, a high degree of sequence homology has been found between *B. asper* myotoxin II and a Lys-49 PLA_2 of *A. p. piscivorus* venom [11]. Other venoms tested, despite containing basic PLA_2s with myotoxic activity (*N. n. atra, C. d. terrificus*), or basic myotoxic peptides (*C. v. viridis*) [33], were not susceptible to heparin neutralization. Further studies using purified PLA_2s/myotoxins from these genera are needed, as the experiments were performed with crude venoms. Nevertheless, these findings suggest that the interaction of heparins with some toxic PLA_2s could be based not only on a non-specific electrostatic interaction due to the basic character of the enzymes, but that some specific recognition component might additionally be involved. The lack of myotoxin II neutralization by chondroitin sulfate is also in agreement with this speculation. The structural nature of the interaction between heparins and myotoxic PLA_2s is currently being investigated.

Interestingly from the medical point of view, the ability of heparin to neutralize the cytolytic action of myotoxins did not depend on its anticoagulant activity, since similar results were obtained with conventional heparin and LA-heparin. This is of relevance since *Bothrops* venoms contain potent hemorrhagic toxins and also severely disturb coagulation [34, 35]. Indeed, the potentiating effect of standard heparin on the hemorrhagic action of the venom was evident in the intravital microscopy experiments, emphasizing the potential risks of its use.

As shown by the electrophoretic analyses, all myotoxin isoforms described in *B. asper* venom [36] could be recovered from a heparin affinity column. Of these, myotoxin II, a Lys-49 PLA_2 isoform, was studied in more detail. Since the muscle damaging effect of these PLA_2s is currently considered to be related to their ability to penetrate phospholipid bilayers and alter cell membrane permeability [37–41], an *in vitro* cytotoxicity system was utilized. Myotoxin II was clearly cytolytic to skeletal muscle myoblasts and capillary endothelial cells, the latter cell type being significantly more susceptible. This was unexpected, as it has recently been described that myotoxin III from the same venom is more toxic to cultured L6 myoblasts than to a variety of other cell types, although endothelial cells were not tested [42]. The ratios at which heparins completely

blocked this cytotoxic effect suggest that several toxin molecules may simultaneously be neutralized by one heparin molecule. This could be explained by the polysaccharide nature of heparin, with its linear repeating unit structure [43]. However, the neutralizing efficiency of heparins in the myotoxicity tests *in vivo* was lower than in the cytotoxicity assay *in vitro*. This could be due to several reasons. One possibility to explain this difference would be a partial dissociation of myotoxin/heparin complexes, due to the competition caused *in vivo* by high affinity heparin-binding factors. On the other hand, although the group of basic PLA_2 myotoxins is the main mediator of muscle damage in this venom [18], it is possible that other factors not affected by heparins, for example acidic hemorrhagic toxins causing ischemia, could contribute to some degree to myonecrosis [33]. Nevertheless, even when using purified myotoxins, the neutralizing efficiency of heparin was lower *in vivo* than *in vitro*. Still another alternative, although at present speculative, explanation is that myotoxins would have a significantly higher affinity for mature muscle fibers than for myoblasts (or other cell types) utilized in culture. Although it is clear that several types of cells can be killed *in vitro* by myotoxic PLA_2s of *Bothrops* venoms [41, 42, and present study], the only cell type reported to undergo necrosis *in vivo* is the mature muscle fiber [37]. Immature muscle cell precursors are likely to be less susceptible to myotoxin action, as suggested by the regeneration of affected muscles [44, 45]. The determination of the binding affinity of myotoxins to heparin, and to diverse cell types in culture, particularly to more differentiated muscle cell stages such as myotubes, would shed light on this hypothesis. However, the results obtained with the neutralizing monoclonal antibody MAb-3, do not agree with the concept of a differential affinity/susceptibility to myotoxins of mature muscle, compared to the cultured cell lines: the neutralizing potency of MAb-3 *in vitro* was similar to that reported *in vivo* [18], with a complete inhibition of myotoxin II achieved at an antibody/toxin molar ratio of about 1/1. Further work is needed to clarify the different neutralizing potency of heparins *in vitro* and *in vivo*.

The possibility of an effect of heparins on the target cells, rather than on the toxin, was clearly excluded. The details of the mechanism of myotoxin neutralization by heparins, remain to be determined. Diccianni *et al.* [3] demonstrated that heparin inhibits the hydrolysis of micellar phospholipid substrates by porcine pancreatic PLA_2, by binding to its amino-terminal region, known to act as the interface recognition site. It would be of interest to determine if heparin binds to an analogous region of myotoxic PLA_2s, thereby interfering with penetration and subsequent perturbation of cell membrane homeostasis.

The interaction of heparin with PLA_2 of different origins has been reported to affect enzymatic activity in some cases [2, 3, 46], but not in others [1, 47, 48]. In the case of myotoxin III, its PLA_2 activity was unaffected by heparins, despite that its myotoxic effect was significantly reduced. Thus, the two toxin actions were clearly dissociated, in agreement with earlier results obtained with neutralizing monoclonal antibodies [18].

LA-heparin was also able to neutralize the edema induced by myotoxin II, in addition to its cytotoxic action. Although the edema-forming effect of PLA_2s has usually been correlated to their ability to hydrolyse phospholipids [49–51], the discovery of Lys-49 PLA_2s devoid of enzymatic activity but still able to elicit edema [10, 52], implies that other mechanisms can also participate in this pharmacological activity. Among these mechanisms might be a direct action on mast cells [52, 53] or on vascular endothelium. In this regard, it is interesting to note that myotoxin II had direct effects on endothelial cells *in vitro*, which were inhibited by heparins. However, since the exact mechanisms by which Lys-49 PLA_2s induce edema *in vivo* are not known, it is difficult to speculate about the mode of heparin neutralization of this effect. Indeed, it would be reasonable to consider the possibility that the edema induced by myotoxin II would be a response to the rapid muscle fiber damage [15], and that neutralization of myotoxicity by heparins would result in an inhibition of this response. However, the observation that some venoms potently induce myonecrosis without a detectable edema response [54] argues against this hypothesis. A similar inhibitory activity of heparin on the edema induced by a PLA_2 from *Trimeresurus mucrosquamatus* has been reported [53].

The ability of free heparan sulfate to block the cytolytic activity of myotoxin II led us to investigate the possible role of cell surface heparan sulfate proteoglycan in the cytotoxic mechanism of these toxins, either as a protecting factor or as a first receptor/susceptibility factor, since their cell binding site(s) have not yet been identified. The results obtained by a combination of approaches, including heparitinase and chlorate cell treatments, as well as the use of CHO-*pgs*D-677 mutant cell line, all indicated that cell surface heparan sulfate is not required by these toxins to exert their cytolytic action.

The present results suggest that the potential of LA-heparin, or even smaller heparin fragments devoid of anticoagulant effects, should be experimentally evaluated as a possible aid in the treatment of snakebites from certain species. Bergonzini *et al.* [55] demonstrated that bioavailability and distribution of heparins in biological compartments depend on their molecular weight. The screening of different heparin-derived disaccharides showed that the neutralizing effect cannot be achieved with such small structures, and therefore requires slightly larger oligosaccharides, as has been shown for the interaction of heparin with other proteins [56]. Thus, the determination of the minimal heparin structure capable to block the cytolytic effect of myotoxic PLA_2s, possibly with a faster tissue distribution than antibodies [57], will point out candidate compounds to be evaluated in future studies.

Acknowledgements—We thank Margareta Verdrengh, Ing-Marie Nilsson and Lena Svensson for excellent laboratory support, Prof. Ulf Lindahl (Uppsala University) for valuable advice and cooperation, and Drs J. D. Esko, M.

Thelestam, A. Mantovani, J. M. Gutiérrez, L. O. Andersson, M. Maccarana, E. Hurt and E. Trybala for providing valuable materials. This work was supported by the Swedish Agency for Research and Cooperation with Developing Countries (SAREC), Kabi Pharmacia, Consejo Nacional de Investigaciones Científicas y Tecnológicas de Costa Rica (CONICIT), the International Foundation for Science (F/1388-2) and the Swedish Medical Research Council (00663).

REFERENCES

1. Horigome K, Hayakawa M, Inoue K and Nojima S, Purification and characterization of phospholipase A_2 released from rat platelets. *J Biochem* **101**: 625–631, 1987.
2. Diccianni MB, Mistry MJ, Hug K and Harmony JAK, Inhibition of phospholipase A_2 by heparin. *Biochim Biophys Acta* **1046**: 242–248, 1990.
3. Diccianni MB, Lilly M, McLean LR, Balasubramaniam A and Harmony JAK, Heparin prevents the binding of phospholipase A_2 to phospholipid micelles: importance of the amino-terminus. *Biochemistry* **30**: 9090–9097, 1991.
4. Kini RM and Evans HJ, A model to explain the pharmacological effects of snake venom phospholipases A_2. *Toxicon* **27**: 613–635, 1989.
5. Davidson FF and Dennis EA, Evolutionary relationships and implications for the regulation of phospholipase A_2 from snake venoms to human secreted forms. *J Mol Evol* **31**: 228–238, 1990.
6. Gutiérrez JM, Chaves F, Bolaños R, Cerdas L, Rojas E, Arroyo O and Portilla E, Neutralización de los efectos locales del veneno de *Bothrops asper* por un antiveneno polivalente. *Toxicon* **19**: 493–500, 1981.
7. Ownby CL, Odell GV, Woods WM and Colberg TR, Ability of antiserum to myotoxin *a* from prairie rattlesnake (*Crotalus viridis viridis*) venom to neutralize local myotoxicity and lethal effects of myotoxin and homologous crude venom. *Toxicon* **21**: 35–45, 1983.
8. Melo PA and Suarez-Kurtz G, Release of sarcoplasmic enzymes from skeletal muscle by *Bothrops jararacussu* venom: antagonism by heparin and by the serum of South American marsupials. *Toxicon* **26**: 87–95, 1988.
9. Melo PA, Homsi-Brandeburgo MI, Giglio JR and Suarez-Kurtz G, Antagonism of the myotoxic effect of *Bothrops jararacussu* venom and bothropstoxin by polyanions. *Toxicon* **31**: 285–291, 1993.
10. Lomonte B and Gutiérrez JM, A new muscle damaging toxin, myotoxin II, from the venom of the snake *Bothrops asper* (terciopelo). *Toxicon* **27**: 725–733, 1989.
11. Francis B, Gutiérrez JM, Lomonte B and Kaiser II, Myotoxin II from *Bothrops asper* (terciopelo) venom is a lysine-49 phospholipase A_2. *Arch Biochem Biophys* **284**: 352–359, 1991.
12. Kaiser II, Gutiérrez JM, Plummer D, Aird SD and Odell GV, The amino acid sequence of a myotoxic phospholipase from the venom of *Bothrops asper*. *Arch Biochem Biophys* **278**: 319–325, 1990.
13. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
14. Reisfeld RA, Lewis UJ and Williams DE, Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195**: 281–283, 1962.
15. Lomonte B, Lundgren J, Johansson B and Bagge U, The dynamics of local tissue damage induced by *Bothrops asper* snake venom and myotoxin II on the mouse cremaster muscle: an intravital and electron microscopic study. *Toxicon* **32**: 41–55, 1994.
16. Bussolino F, De Rossi M, Sica A, Colotta F, Wang JM, Bocchietto E, Padura IM, Bosia A, Dejana E and Mantovani A, Murine endothelioma cell lines transformed by polyoma middle T oncogene as target for and producers of cytokines. *J Immunol* **147**: 2122–2129, 1991.
17. Lomonte B and Kahan L, Production and partial characterization of monoclonal antibodies to *Bothrops asper* (terciopelo) myotoxin. *Toxicon* **26**: 675–689, 1988.
18. Lomonte B, Gutiérrez JM, Ramírez M and Díaz C, Neutralization of myotoxic phospholipases A_2 from the venom of the snake *Bothrops asper* by monoclonal antibodies. *Toxicon* **30**: 239–245, 1992.
19. Mancini G, Carbonara AO and Heremans JF, Immunochemical quantitation of antigens by radial immunodiffusion. *Immunochemistry* **2**: 235–254, 1965.
20. Lomonte B, Tarkowski A and Hanson LÅ, Host response to *Bothrops asper* snake venom: analysis of edema formation, inflammatory cells, and cytokine release in a mouse model. *Inflammation* **1**: 93–105, 1993.
21. Gutiérrez JM, Avila C, Rojas E and Cerdas L, An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **26**: 411–413, 1988.
22. Ouchterlony Ö and Nilsson LÅ, Immunodiffusion and immunoelectrophoresis. In: *Handbook of Experimental Immunology* (Ed. Weir DM), pp. 19.1–19.44. Blackwell Scientific Publications, Oxford, 1978.
23. WuDunn D and Spear PG, Initial interaction of Herpes Simplex virus with cells is binding to heparan sulfate. *J Virol* **63**: 52–58, 1989.
24. Greve H, Cully Z, Blumberg P and Kresse H, Influence of chlorate on proteoglycan biosynthesis by cultured human fibroblasts. *J Biol Chem* **263**: 12886–12892, 1988.
25. Olwin BB and Rapraeger A, Repression of myogenic differentiation by aFGF, bFGF, and K-FGF is dependent on cellular heparan sulfate. *J Cell Biol* **118**: 631–639, 1992.
26. Lidholt K, Weinke JL, Kiser CS, Lugemwa FN, Bame KJ, Cheifetz S, Massagué J, Lindahl U and Esko JD, A single mutation affects both *N*-acetylglucosaminyltransferase and glucuronosyltransferase activities in a Chinese hamster ovary cell mutant defective in heparan sulfate biosynthesis. *Proc Natl Acad Sci USA* **89**: 2267–2271, 1992.
27. Lomonte B, Furtado MF, Rovira ME, Carmona E, Rojas G, Aymerich R and Gutiérrez JM, South American snake venom proteins antigenically-related to *Bothrops asper* myotoxins. *Braz J Med Biol Res* **23**: 427–435, 1990.
28. Cintra ACO, Marangoni S, Oliveira B and Giglio JR, Bothropstoxin-I: amino acid sequence and function. *J Protein Chem* **12**: 57–64, 1993.
29. Lomonte B, Gutiérrez JM, Furtado MF, Otero R, Rosso JP, Vargas O, Carmona E and Rovira ME, Isolation of basic myotoxins from *Bothrops moojeni* and *Bothrops atrox* snake venoms. *Toxicon* **28**: 1137–1146, 1990.
30. Kihara H, Uchikawa R, Hattori S and Ohno M, Myotoxicity and physiological effects of three *Trimeresurus flavoviridis* phospholipases A_2. *Biochem Int* **28**: 895–903, 1992.
31. Díaz C, Gutiérrez JM and Lomonte B, Isolation and characterization of basic myotoxic phospholipases A_2 from *Bothrops godmani* (Godman's pitviper) snake venom. *Arch Biochem Biophys* **298**: 135–142, 1992.
32. Lomonte B, Moreno E and Gutiérrez JM, Detection of proteins antigenically-related to *Bothrops asper* myotoxin in crotaline snake venoms. *Toxicon* **25**: 947–955, 1987.
33. Ownby CL, Locally acting agents: myotoxins, hemorrhagic toxins and dermonecrotic factors. In: *Hand-*

book of Toxinology (Eds. Shier WT and Mebs D), pp. 601–654. Marcel Dekker, New York, 1990.
34. Gutiérrez JM and Lomonte B, Local tissue damage induced by *Bothrops* snake venoms: a review. *Memorias Inst Butantan* **51**: 211–223, 1989.
35. Tan NH and Ponnudurai G, A comparative study of the biological properties of some venoms of snakes of the genus *Bothrops* (American lance-headed viper). *Comp Biochem Physiol* **100B**: 361–365, 1991.
36. Lomonte B and Carmona E, Individual expression patterns of myotoxin isoforms in the venom of the snake *Bothrops asper*. *Comp Biochem Physiol* **102B**: 325–329, 1992.
37. Gutiérrez JM, Ownby CL and Odell GV, Pathogenesis of myonecrosis induced by crude venom and a myotoxin of *Bothrops asper*. *Exp Mol Pathol* **40**: 367–379, 1984.
38. Díaz C, Gutiérrez JM, Lomonte B and Gené JA, The effect of myotoxins isolated from *Bothrops* snake venoms on multilamellar liposomes: relationship to phospholipase A_2, anticoagulant and myotoxic activities. *Biochim Biophys Acta* **1070**: 455–460, 1991.
39. Rufini S, Cesaroni P, Desideri A, Farias R, Gubensek F, Gutiérrez JM, Luly P, Massoud R, Morero R and Pedersen JZ, Calcium ion independent membrane leakage induced by phospholipase-like myotoxins. *Biochemistry* **31**: 12424–12430, 1992.
40. Bultrón E, Gutiérrez JM and Thelestam M, Effects of *Bothrops asper* (terciopelo) myotoxin III, a basic phospholipase A_2, on liposomes and mouse gastrocnemius muscle. *Toxicon* **31**: 217–222, 1993.
41. Brusés JL, Capaso J, Katz E and Pilar G, Specific *in vitro* biological activity of snake venom myotoxins. *J Neurochem* **60**: 1030–1042, 1993.
42. Bultrón E, Thelestam M and Gutiérrez JM, Effects on cultured mammalian cells of myotoxin III, a phospholipase A_2 isolated from *Bothrops asper* (terciopelo) venom. *Biochim Biophys Acta* **1179**: 253–259, 1993.
43. Kjellén L and Lindahl U, Proteoglycans: structures and interactions. *Annu Rev Biochem* **60**: 443–475, 1991.
44. Gutiérrez JM, Ownby CL and Odell GV, Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake *Bothrops asper* (fer-de-lance). *Toxicon* **22**: 719–731, 1984.
45. Harris JB and Cullen MJ, Muscle necrosis caused by snake venoms and toxins. *Electron Microsc Rev* **3**: 183–211, 1990.
46. Franson R, Patriarca P and Elsbach P, Phospholipid metabolism by phagocytic cells. Phospholipases A_2 associated with rabbit polymorphonuclear leukocyte granules. *J Lipid Res* **15**: 380–388, 1974.
47. Condrea E and de Vries A, Hemolysis and splitting of human erythrocyte phospholipids by snake venoms. *Biochim Biophys Acta* **84**: 60–73, 1964.
48. Ho CL, Tsai IH and Lee CY, The role of enzyme activity and charge properties on the presynaptic neurotoxicity and the contracture-inducing activity of snake venom phospholipases A_2. *Toxicon* **24**: 337–345, 1986.
49. Lloret S and Moreno JJ, Oedema formation and degranulation of mast cells by phospholipase A_2 purified from porcine pancreas and snake venoms. *Toxicon* **31**: 949–956, 1993.
50. Vishwanath BS, Fawzy AA and Franson RC, Edema-inducing activity of phospholipase A_2 purified from human synovial fluid and inhibition by aristolochic acid. *Inflammation* **12**: 549–561, 1988.
51. Cirino G, Peers SH, Wallace JL and Flower RJ, A study of phospholipase A_2-induced oedema in rat paw. *Eur J Pharmacol* **166**: 505–510, 1989.
52. Liu CS, Chen JM, Chang CH, Chen SW, Teng CM and Tsai IH, The amino acid sequence and properties of an edema-inducing Lys-49 phospholipase A_2 homolog from the venom of *Trimeresurus mucrosquamatus*. *Biochim Biophys Acta* **1077**: 362–370, 1991.
53. Wang JP and Teng CM, Comparison of the enzymatic and edema-producing activities of two venom phospholipase A_2 enzymes. *Eur J Pharmacol* **190**: 347–354, 1990.
54. Gutiérrez JM, Lomonte B, Portilla E, Cerdas L and Rojas E, Local effects induced by coral snakes (*Micrurus*) venoms: evidence of myonecrosis after experimental inoculation of venom from five species. *Toxicon* **21**: 777–783, 1983.
55. Bergonzini GL, Bianchini P, Mascellani G, Osima B, Parma B and Volpi N, Different pharmacokinetics, antithrombotic activity and bleeding effects of heparin and two new fragments administered in rat by subcutaneous route. *Arzneim-Forsch/Drug Res* **42**: 1322–1328, 1992.
56. Maccarana M and Lindahl U, Mode of interaction between platelet factor 4 and heparin. *Glycobiology* **3**: 271–277, 1993.
57. Rovira ME, Carmona E and Lomonte B, Immunoenzymatic quantitation of antibodies to *Bothrops asper* myotoxins after polyvalent antivenom administration in mice. *Braz J Med Biol Res* **25**: 23–33, 1992.

VII

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 269, No. 47, Issue of November 25, pp. 29867–29873, 1994
Printed in U.S.A.

Neutralizing Interaction between Heparins and Myotoxin II, a Lysine 49 Phospholipase A_2 from *Bothrops asper* Snake Venom

IDENTIFICATION OF A HEPARIN-BINDING AND CYTOLYTIC TOXIN REGION BY THE USE OF SYNTHETIC PEPTIDES AND MOLECULAR MODELING*

(Received for publication, June 30, 1994, and in revised form, September 8, 1994)

Bruno Lomonte‡§¶, Ernesto Moreno‖, Andrej Tarkowski‡ ‡‡, Lars Å. Hanson‡, and Marco Maccarana§§**

*From the Departments of ‡Clinical Immunology, ‖Medical Biochemistry, and ‡‡Rheumatology, University of Göteborg, Göteborg, Sweden, the §Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica, the **Center of Molecular Immunology, Havana, Cuba, and the §§Department of Medical and Physiological Chemistry, Uppsala Biomedical Center, Uppsala University, Uppsala, Sweden*

Heparin binds to phospholipase A_2 myotoxins from *Bothrops asper* snake venom, inhibiting their toxic activities. This interaction was investigated using purified myotoxin II, a Lys-49 phospholipase A_2 of this venom, and a series of heparin variants, fragments, and other glycosaminoglycans. The binding was correlated to toxin neutralization, using endothelial cells as a target. Myotoxin II binds radiolabeled heparin in solution unselectively, and forms macromolecular complexes with an optimum at a heparin:toxin molar ratio of 1:5. Both *O*-sulfates and *N*-sulfates play a role in heparin binding, in the order of importance 2-*O*-sulfates > 6-Osulfates > *N*-sulfates. The shortest heparin oligosaccharides interacting with myotoxin II are hexasaccharides. The binding of a neutralizing monoclonal antibody (MAb-3) to myotoxin II was not inhibited by heparin, indicating that the two molecules interact with different sites on the toxin. A synthetic peptide (residues 115–129 in the numbering system of Renetseder *et al.* (Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., and Sigler, P. B. (1985) *J. Biol. Chem.* 260, 11627–11634) of myotoxin II displays both heparin-binding and cytolytic activities. It is concluded that heparin neutralizes myotoxin II by binding to a strongly cationic site in the region of residues 115–129, a possible contribution of lysines 36 and 38 suggested by molecular modeling studies. As this cationic region appears to be responsible for the cytolytic activity of the toxin, the present report constitutes the first identification of a cytotoxic region on a phospholipase A_2 myotoxin.

Phospholipases A_2 (PLA_2s; EC 3.1.1.4)[1] in snake venoms have acquired a variety of pharmacological/toxic activities, including neurotoxicity, myotoxicity, anticoagulant effect, and inflammatogenicity (1). Although their toxic effects have been *a priori* conceived as being consequence of the enzymatic activity, a growing body of evidence indicates that some toxic actions do not depend on phospholipolytic activity (1, 2). In the case of non-neurotoxic PLA_2 myotoxins, clear examples of the lack of requirement of an intrinsic enzymatic activity for the induction of muscle necrosis (3–7) or anticoagulant effect (8) have been presented. Nevertheless, the molecular site responsible for the toxic activity of PLA_2 myotoxins has remained elusive (1).

Heparin is a sulfated glycosaminoglycan (GAG) composed by alternating hexuronic (glucuronic (GlcA) or iduronic (IdoA) acid and glucosamine (GlcN) units. Sulfate substituents are mainly *N*-sulfate groups (at C-2 position of GlcN) or *O*-sulfate groups (at C-6 of GlcN and C-2 of IdoA) (9). A previous study showed that both standard heparin and heparin with low affinity for antithrombin bind to myotoxic PLA_2s from the venom of *Bothrops asper*, inhibiting their *in vitro* cytolytic and *in vivo* myotoxic activities (10). Neutralization of myotoxic activity of the venoms from at least three other taxa containing PLA_2s structurally related to those of *B. asper* was also observed (10). Thus, heparin and its derivatives, especially those with little or no anticoagulant activity, might have potential as a complementary treatment of envenomations caused by several species of snakes.

The purpose of the present work was (i) to investigate some basic aspects of this protein-GAG interaction, and (ii) to approach the molecular mechanism of myotoxin neutralization, through the search for a heparin-binding site. For these purposes, we utilized purified myotoxin II, a Lys-49 phospholipase A_2 of *B. asper* (5, 11), synthetic peptides, and a series of heparin variants, fragments, and other sulfated GAGs. Special interest was given to the correlation between the binding interaction and the neutralization of cytolytic activity. Use of synthetic peptides helped to identify a myotoxin region involved both in interaction with heparin, and in cytolytic action. Experimental data were complemented by molecular modeling studies of the proposed heparin-binding and cytolytic region.

EXPERIMENTAL PROCEDURES

Glycosaminoglycans—Heparin from pig intestinal mucosa (stage 14, Inolex Pharmaceutical Division) was purified as described elsewhere (12) and radiolabeled by ^{3}H-acetylating free amino groups through treatment with labeled acetic anhydride (13). The product had a specific activity of $\sim 0.6 \times 10^6$ dpm/nmol, assuming a molecular weight of 15,000.

6-*O*-Desulfation along with *N*-desulfation of heparin was achieved by treatment with dimethyl sulfoxide, 10% water at 110 °C for 5 h (14), and was followed by either re-*N*-sulfation (15) or *N*-acetylation (16). Compositional analysis of the re-*N*-sulfated product indicated non-*O*-sulfated-HexA-$GlcNSO_3$ (39%) and IdoA(2-OSO_3)-$GlcNSO_3$ (46%) as the

* This study was supported by the Swedish Agency for Research Cooperation with Developing Countries (SAREC), Kabi-Pharmacia (Stockholm, Sweden), Consejo Nacional para Investigaciones Científicas y Tecnológicas de Costa Rica (CONICIT), International Foundation for Science (F/1388–2), Swedish Medical Research Council Grant 2309, and Polysackaridforskning AB (Uppsala, Sweden). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed. Tel.: 506-229-0344; Fax: 506-292-0485.

[1] The abbreviations used are: PLA_2, phospholipase A_2; GAG, glycosaminoglycan; HexA, unspecified hexuronic acid; GlcN, 2-amino-2-deoxyglucose (D-glucosamine); IdoA, L-iduronic acid; GlcA, D-glucuronic acid; PBS, phosphate-buffered saline; r.m.s., root mean square.

predominant disaccharide units. 2-*O*-Desulfated heparin was obtained under alkaline conditions (17) and contained 21% non-*O*-sulfated HexA-GlcNSO$_3$ and 70% IdoA-GlcNSO$_3$(6-OSO$_3$) as the predominant disaccharide units.

Selective *N*-desulfation was obtained by treatment with dimethyl sulfoxide:H_2O 19:1 at 50 °C (18). The sample, quantitatively retaining *O*-sulfate groups, was completely *N*-acetylated.

O- and *N*-desulfated heparin was prepared by treatment with 10% methanol in dimethyl sulfoxide at 100 °C for 2 h (14). The product was completely re-*N*-sulfated or completely *N*-acetylated. In both cases the preparations still retained ~0.3 *O*-sulfate group/disaccharide unit located exclusively in position C-2 of the iduronic acid.

A low sulfated heparan sulfate (~0.6 sulfate group/disaccharide unit) from human aorta (19) was provided by W. Murphy (University of Monash, Australia). Chondroitin sulfate (bovine cartilage) and dermatan sulfate (pig intestinal mucosa) were obtained as described previously (20).

Oligosaccharides—Even-numbered heparin oligosaccharides were generated by partial deaminative cleavage of the polysaccharide with nitrous acid (pH 1.5; cleavage at *N*-sulfated GlcN units) (21), essentially as described elsewhere (22, 23), and the resulting 2,5-anhydro-D-mannose residues were reduced with either $NaBH_4$ or NaB^3H_4 (Amersham Corp.). The resulting labeled oligosaccharides had a specific activity of ~0.6 × 10^6 dpm ^{3}H/nmol. Specific activities of heparin and heparin-oligosaccharides were expressed on the basis of hexuronic acid content, as measured by the carbazole reaction (24).

Purification of Myotoxin II and Preparation of Synthetic Peptides—Myotoxin II was purified from the venom of *B. asper* (5), appearing as a single band of 15,000 Da in SDS-polyacrylamide gel electrophoresis (25) and cathodic native polyacrylamide gel electrophoresis (26).

Three peptides, corresponding to residues 1–26 (SLFELGKMILQETGKNPAKSYGAYGC), 60–71 (KKDRYSYSWKDK), and 105–117 (KKYRYYLKPLCKK) of myotoxin II, respectively, with native endings, were synthesized by Chiron Mimotopes (Victoria, Australia) using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) strategy (27). In the numbering sytem of Renetseder *et al.* (28), these peptides correspond to residues 1–27, 69–80, and 115–129, respectively. Their purity was at least 96, 75, and 96%, respectively, as assessed by high performance liquid chromatography on a LiCrosphere 100RP-18 column or by mass spectrometry. The sequences were based on data from Francis *et al.* (11).

Interaction between Saccharides and Myotoxin II—Myotoxin II was incubated at room temperature for 2 h with the appropiate saccharides in 200 or 300 µl of 50 mM Tris-HCl, pH 7.4, 130 mM NaCl (TBS) containing 0.5 mg/ml of bovine serum albumin. The protein, along with any bound GAGs, was recovered by quick passage of the mixtures through nitrocellulose filters (Sartorius, pore size 0.45 µm; 25-mm diameter) which had been placed onto a 10-well vacuum-assisted manifold filtration apparatus. The filters were prewashed twice with 5 ml of TBS, before application of samples, which were immediately followed by another two washings with the same buffer; each washing step was completed within 5 s. Protein-bound radioactivity was determined after submersion of the filters in 2 ml of 2 M NaCl for 30 min; the filters were withdrawn and the eluate was mixed with 2 ml of water and 12 ml of scintillation mixture (OptiPhase, Pharmacia Biotech Inc.) and counted in a Beckman LS 6000IC scintillation spectrometer. No residual radioactivity could be detected on the filters (for reference, see Maccarana *et al.* (29)). The variation between duplicates was generally <5%.

Cytotoxicity Assay—The cytotoxic activity of myotoxin II, and the neutralizing effect of GAGs, were quantified on an endothelial cell line (tEnd) (30) as previously described (10). Cells were grown in 96-well plates until near confluence, in Iscove's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5 × 10^{-5} M 2-mercaptoethanol, and 0.05 mg/ml gentamycin. At the time of the assay, culture medium was replaced with 150 µl/well of medium with 1% fetal calf serum, containing 10 µg of myotoxin II, which had been previously incubated in the same medium (15 min, room temperature) with each GAG. The fetal calf serum was lowered to 1% to minimize the basal lactate dehydrogenase (EC 1.1.1.27) activity of the medium. After 3 h of incubation at 37 °C, 100 µl of supernatant were assayed for lactate dehydrogenase (kit no. 500, Sigma). For 100 and 0% cytotoxicity values, cells were incubated with 0.1% Triton X-100-containing medium or plain medium, respectively. Control samples of GAGs without toxin were included. The cytotoxic activity of peptides, dissolved either in Iscove's medium or phosphate-buffered saline (PBS; 0.12 M NaCl, 40 mM sodium phosphate, pH 7.2), was determined similarly.

Enzyme Immunoassay for Competition Binding of MAb-3—MAb-3, a monoclonal IgG_1 antibody (31) neutralizing the myotoxic (32) and cytolytic (10) activities of myotoxin II, was utilized to determine if heparin competed for its binding to myotoxin. The competition was assessed by incubating (i) a fixed amount of heparin with varying amounts of MAb-3, and (ii) a fixed amount of MAb-3 with varying amounts of heparin. Different heparin/MAb-3 mixtures, in Iscove's medium with 1% fetal calf serum, were applied to microwells coated with myotoxin II (0.4 µg/well), and incubated for 4 h at room temperature. After five washings with PBS containing 0.05% Tween 20, MAb-3 was detected with anti-mouse IgG-horseradish peroxidase (Cappel). Color was developed with hydrogen peroxide and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid), and recorded at 405 nm. Horse polyvalent antivenom (batch 203LQ, Instituto Clodomiro Picado) (33) was utilized as a positive control for competition in the binding of MAb-3 to myotoxin II.

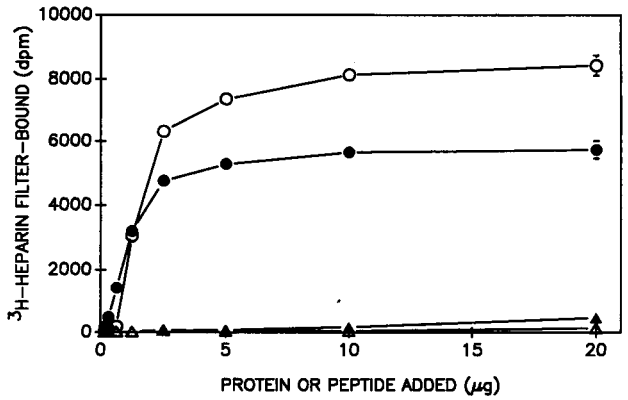

FIG. 1. **Binding of ^{3}H-labeled heparin to myotoxin II and synthetic peptides.** Approximately 10,000 dpm of ^{3}H-labeled heparin (~90 nM) was added to increasing amounts of myotoxin II (5 µg, for instance, corresponding to 1.7 µM), or peptides, in a final volume of 200 µl of incubation buffer. The amounts of ^{3}H-labeled filter associated with myotoxin II or peptides were determined by the filtration procedure through nitrocellulose described under "Experimental Procedures." Blank values without protein/peptides gave 80 dpm. (○) myotoxin II; (●) peptide 115–129; (△) peptide 1–27; (▲) peptide 69–80. Values are means of duplicate assays ± S.D. If not represented, S.D. is smaller than symbol size.

Molecular Modeling Analyses—Molecular modelings were conducted on a Silicon Graphics Indigo^2 Extreme workstation using QUANTA/CHARMm 3.3 and Biograf softwares (Molecular Simulations). A computer model of myotoxin II was constructed using as starting geometry the crystal structure of *Agkistrodon piscivorus piscivorus* K49 (34), available from the Brookhaven Protein Data Bank. Side chain replacements were made to the *A. p. piscivorus* K49 structure, followed by an energy minimization of the whole molecule to an r.m.s. tolerance of the force field gradient of 0.1 kcal/mol·Å using CHARMm (ϵ = 8, *r*-dependent).

A model of peptide 115–129 was also constructed by extracting its coordinates from the minimized structure of myotoxin II. The peptide was surrounded by a water shell (outer cutoff 9 Å) using Biograf. The water was first minimized to an r.m.s. tolerance of the force field gradient of 0.05 kcal/mol·Å using CHARMm (ϵ = 1) while keeping the peptide fixed, whereafter the whole structure including the water was minimized again down to the same r.m.s. value. A molecular dynamics simulation was then conducted for the complex peptide-water. The system was heated to 300 K for 6 ps and equilibrated at this temperature for another 6 ps, and finally a 50-ps microcanonical simulation was run. All bond lengths were kept constant during the simulation using the SHAKE algorithm, allowing a time step of 2 fs.

RESULTS

Myotoxin II—The direct binding of ^{3}H-labeled heparin to increasing amounts of myotoxin II is shown in Fig. 1. Most of the added heparin was bound by the protein readily at 5 µg of myotoxin II/assay. The ability of different unlabeled GAGs to interact with myotoxin II was studied by competition binding with ^{3}H-labeled heparin, as shown in Fig. 2*A* and Table I. Notably, the *N*-desulfated *N*-acetylated heparin was as efficient as the unmodified heparin in the binding. Selective *O*-desulfation of heparin resulted in a moderate decrease in the interaction, approximately 2-fold for 6-*O*-desulfated heparin, and 6-fold in the case of 2-*O*-desulfated heparin. The interaction

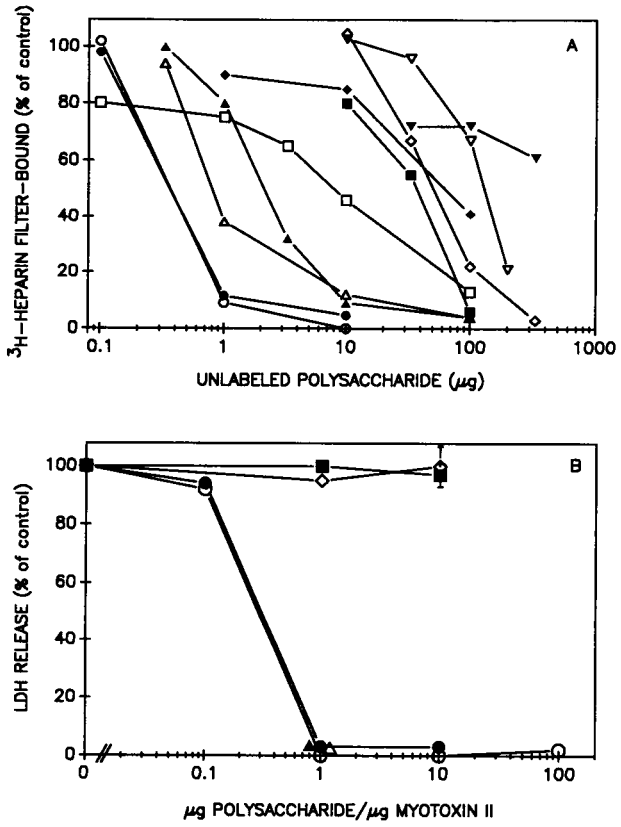

FIG. 2. **Interaction of heparins and other glycosaminoglycans with myotoxin II.** *A*, competitive direct binding to myotoxin II of ^{3}H-labeled heparin and unlabeled GAGs. 300-μl incubation mixtures containing 2.5 μg of myotoxin II (555 nM), 1×10^4 dpm of ^{3}H-labeled heparin (~60 nM), and various amounts of unlabeled GAGs (1 μg of heparin, for instance, corresponding to 222 nM) were passed through nitrocellulose filters as described under "Experimental Procedures." Controls without adding unlabeled polysaccharide showed 5,200 dpm bound to myotoxin II and retained by the filters; blanks without myotoxin II gave 60 dpm. The unlabeled polysaccharides were heparin (○), *N*-desulfated *N*-acetylated heparin (●), 6-*O*-desulfated heparin (△), 2-*O*-desulfated heparin (▲), 2- and 6-*O*-desulfated heparin (□), human aorta heparan sulfate (■), chondroitin sulfate (◇), 6-*O*-desulfated *N*-acetylated heparin (◆), dermatan sulfate (▽), and *O*- and *N*-desulfated *N*-acetylated heparin (▼). *B*, effect of heparins and other glycosaminoglycans on the cytotoxic activity of myotoxin II to endothelial cells (tEnd). Myotoxin II (10 μg/150 μl/well), either alone or after incubation with heparins or other GAGs for 15 min at room temperature, was applied to cell cultures. Cytotoxicity was determined by the release of lactic dehydrogenase to the medium after 3 h at 37 °C, as described under "Experimental Procedures." Lactate dehydrogenase release is expressed as a percentage, considering as 100% the activity of control cultures treated with 0.1% Triton X-100. Heparin (○), *N*-desulfated *N*-acetylated heparin (●), 6-*O*-desulfated heparin (△), 2-*O*-desulfated heparin (▲), human aorta heparan sulfate (■), and chondroitin sulfate (◇). The two selectively *O*-desulfated heparin samples were tested only at a single ratio (1 μg/μg toxin).

was even weaker with the heparin desulfated at both 2-*O* and 6-*O* positions, and with a low sulfated human aorta heparan sulfate. Dermatan sulfate and chondroitin sulfate competed poorly in the binding, only at concentrations about two orders of magnitude higher than that of unmodified heparin.

Neutralization of the cytolytic activity of myotoxin II conformed to the binding inhibition data. Titration with the unmodified heparin indicated complete neutralization of toxin activity at a ratio of ≥1 μg of GAG/μg protein (Fig. 2*B*). *N*-Desulfated *N*-acetylated heparin, 2-*O*- and 6-*O*-desulfated heparins, all neutralized the toxin at this ratio, while no inhibitory effect was observed with chondroitin sulfate or low-sulfated heparan sulfate, even at GAG/protein mass ratios 10 times higher.

In direct binding of ^{3}H-labeled oligosaccharides to myotoxin II, the smallest heparin fragment capable of interaction was the hexasaccharide, the level of binding increasing with saccharide length (Fig. 3*A*). Accordingly, tests of unlabeled oligosaccharides for myotoxin neutralization showed that hexasaccharides provided partial protection, octasaccharides almost complete protection, and decasaccharides or larger species full protection against the cell-damaging activity of the toxin (Fig. 3*B*).

Macromolecular complex formation was investigated by adding increasing amounts of heparin to a myotoxin II solution, followed by measurement of the turbidity at 340 nm (Fig. 4*A*). Myotoxin II did not absorb light at this wavelength. Maximal turbidity was obtained at a ratio of 0.2 μg of heparin/μg of protein (5 protein molecules/heparin chain), and decreased at further additions of heparin. In the filter assay, when increasing amounts of ^{3}H-labeled heparin were added to a fixed amount of myotoxin II, the maximal retention occurred at a heparin:toxin molar ratio of 1:10, and then decreased as the proportion of heparin increased (Fig. 4*B*).

Heparin, even at very high concentrations, did not inhibit the binding of MAb-3 to myotoxin II (Fig. 5, *A* and *B*). As a control, a polyclonal antivenom preparation competed with MAb-3 in a dose-dependent mode.

Peptides—Of the three synthetic peptides, two (1–27 and 69–80) did not bind heparin in the filter assay (Fig. 1), although they could bind to nitrocellulose as determined by protein staining of dot-blots. In contrast, peptide 115–129 showed considerable heparin-binding ability, comparable to myotoxin II on a mass basis; on a molar basis, about 5–10 times more peptide was required to attain maximal heparin binding (Fig. 1).

Different modified heparins were tested for their ability to displace labeled native heparin from binding to peptide 115–129. Only native unlabeled heparin showed significant displacing ability, all other modified heparins, heparan sulfate, chondroitin and dermatan sulfate, being at least 100 times less potent (data not shown).

When peptide 115–129 was incubated with ^{3}H-labeled heparin oligosaccharides, the shortest oligosaccharides showing a detectable interaction were decasaccharides, the level of binding increasing with increasing saccharide size (data not shown).

In the cytotoxicity system, peptide 115–129 reversed the inhibition induced by heparin, but neither peptide 69–80 (Fig. 6) nor peptide 1–27 (not shown) did. At high concentrations, peptide 115–129 produced a clear cytolytic action *per se*, which was enhanced if the assay was performed using PBS instead of culture medium (Fig. 7). The cytotoxic effect of peptide 115–129 was reproduced using two independently synthesized batches, and was completely abolished by preincubation with heparin (2.4 μg peptide/μg heparin) (Fig. 7). In contrast, peptides 1–27 or 69–80, at the same concentrations, did not induce any cell damage (Fig. 7).

The minimized structure of the myotoxin II model (Fig. 8) showed very small differences as compared with the starting *A. p. piscivorus* K49 crystal structure (r.m.s. value of 0.78 Å for main chain atoms). Dynamics simulations for peptide 115–129, cut out from the native protein, showed that its original backbone conformation was conserved (maximum r.m.s. deviation as compared with the minimized starting structure was 2.52 Å).

DISCUSSION

Some structural characteristics of the interaction between heparin and myotoxin II were studied, and correlated to the neutralization of cytolytic activity. Myotoxin II bound most of the radiolabeled heparin added to the solutions, suggesting a

TABLE 1
Amount of GAGs required to inhibit 50% of ^{3}H-labeled heparin binding to myotoxin II
The values (IC_{50}) are calculated from Fig. 2A.

Glycosaminoglycan	Sulfate groups/disaccharide unit			IC_{50}	Potency
	Total	*N*-Sulfates	*O*-Sulfates		
				μg	% of heparin
Heparin	2.5	1	1.5	0.35	100
N-Desulfated *N*-acetylated heparin	1.5		1.5	0.35	100
6-*O*-Desulfated heparin	1.7	1	0.7	0.8	44
2-*O*-Desulfated heparin	1.8	1	0.8	2	17
2- and 6-O-desulfated heparin	1.3	1	0.3	8	4
Human aorta heparan sulfate	0.6	0.4	0.2	35	1
Chondroitin sulfate	1		1	50	0.7
6-*O*-Desulfated *N*-acetylated heparin	0.7		0.7	70	0.5
Dermatan sulfate	1		1	120	0.3
O- and *N*-desulfated *N*-acetylated heparin	0.3		0.3	>330	<0.1

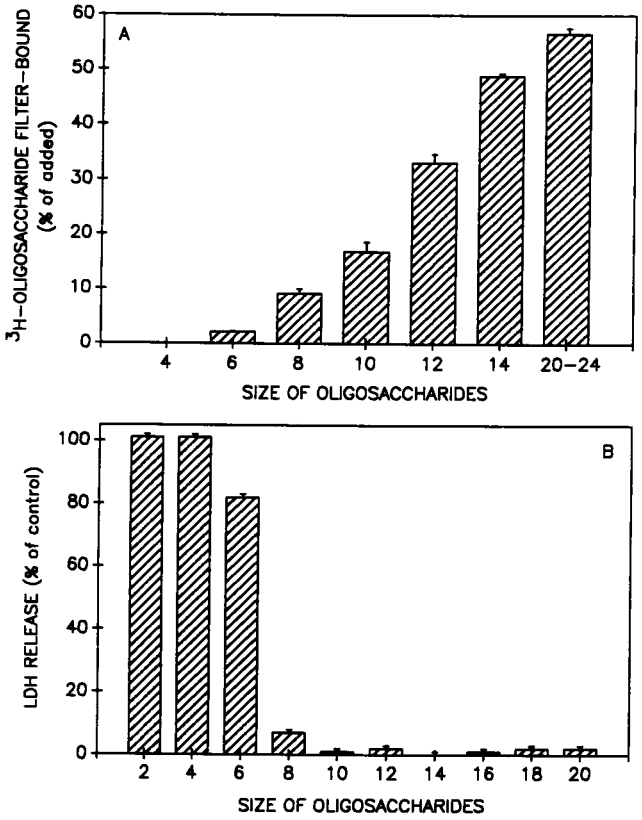

FIG. 3. **Interaction of heparin-derived oligosaccharides with myotoxin II.** *A*, direct binding of labeled oligosaccharides to myotoxin II. ~1 × 10^4 dpm of ^{3}H-labeled oligosaccharides (~60 nM) were incubated with 10 μg of myotoxin II (2.2 μM) in 300 μl of incubation buffer. The amounts of ^{3}H-labeled filter associated with myotoxin II were determined by the nitrocellulose filtration procedure described under "Experimental Procedures." Values are means of duplicate assays ± S.D. and are expressed as percent of the radioactivity added to the incubation mixtures. *B*, neutralization of cytotoxic activity of myotoxin II by unlabeled oligosaccharides. Heparin oligosaccharides of different size were incubated with myotoxin II (15 min at room temperature) at a fixed ratio of 1 μg/μg, and then added to endothelial cell cultures. Cytotoxicity was determined by the release of lactate dehydrogenase, as in the legend for Fig. 2*B*. Values (mean ± S.D. of duplicate assays) are expressed in relation to a control containing myotoxin II (10 μg/150 μl/well) alone.

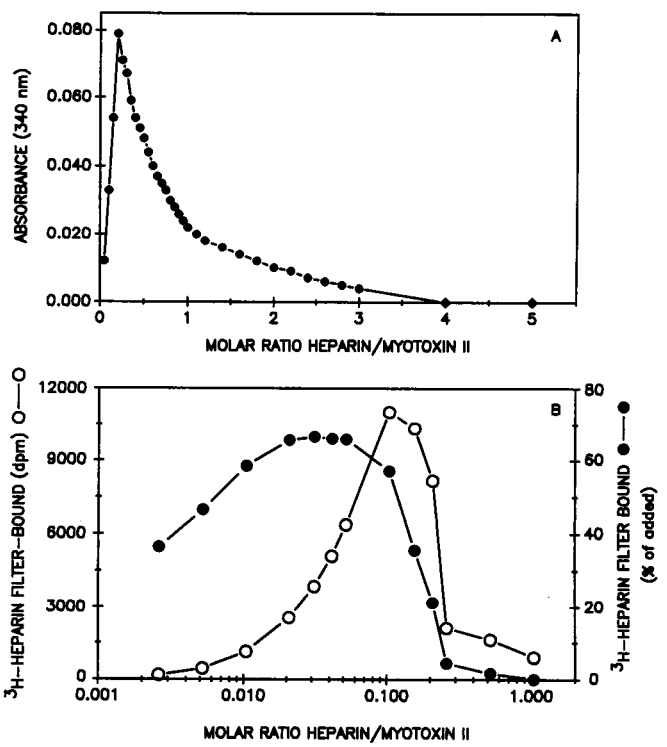

FIG. 4. **Formation of macromolecular complexes between heparin and myotoxin II.** *A*, turbidity at different heparin/myotoxin II ratios. Thirty μg of myotoxin II (2 μM) were added to 2 ml of TBS, followed by additions of 5 μl of concentrated heparin solution to give the final indicated ratio of heparin/myotoxin II; after each addition the mixtures were incubated for 1 min before reading the absorbance at 340 nm. *B*, Binding to the nitrocellulose filter at different heparin/myotoxin II ratios. Increasing amounts of ^{3}H-labeled heparin (from 500 to 200,000 dpm) were added to 5 μg of myotoxin II (1.1 μM) in 300 μl of incubation buffer, to give the final molar ratios of heparin/myotoxin indicated. The amounts of [^{3}H]heparin filter-associated with myotoxin were determined by the nitrocellulose filtration procedure described under "Experimental Procedues." Values are means of duplicate assays.

non-selective mode of interaction, rather than a preferential binding to rare heparin sequences (*i.e.* as antithrombin; Ref. 35). The relative importance of the different sulfate groups was 2-*O*-sulfates > 6-*O*-sulfates > *N*-sulfates. In fact, at the *O*-sulfate density present in unmodified heparin, the *N*-sulfates are redundant for the interaction. However, *N*-sulfates are essential when the *O*-sulfates are decreased to 50% or less of the level present in the native molecule. Two selectively *O*-desulfated heparins showed comparable ability to bind myotoxin, indicating that there is no specific requirement for the position of the *O*-sulfates. Nevertheless, the sample retaining 2-*O* sulfate groups showed a slightly better binding ability compared to the sample having the same overall sulfation, but retaining 6-*O* sulfates. This could be explained by the notion that iduronic acid confers supplementary conformational flexibility to the molecule (36), such that *O*-sulfate groups carried by iduronic acid could fit better to the protein binding site(s) than *O*-sulfate groups carried by glucosamine. The binding of chondroitin sulfate and dermatan sulfate to myotoxin II was weaker

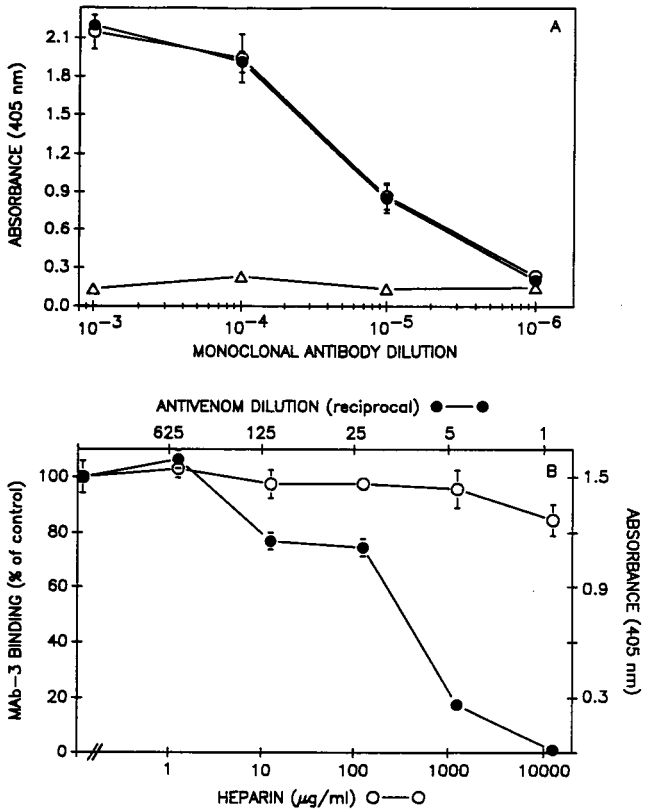

FIG. 5. **Lack of competition between heparin and MAb-3 for binding to myotoxin II in enzyme immunoassay.** *A*, different amounts of MAb-3 were added to myotoxin-coated microplates, either in the absence (○) or the presence (●) of a constant amount (2.5 mg/ml) of heparin. Bound MAb-3 was detected with an anti-mouse IgG-horseradish peroxidase conjugate. Normal mouse serum (△) was included as a control. Absorbance readings are mean ± S.D. of triplicates. *B*, a constant amount of MAb-3 (1:20,000 dilution, selected from the curve in *A*) was added to myotoxin-coated microplates, in the presence of varying amounts of heparin (○). Varying dilutions of a polyclonal horse antivenom (●) were included as a control for competitive binding. Bound MAb-3 was detected as above.

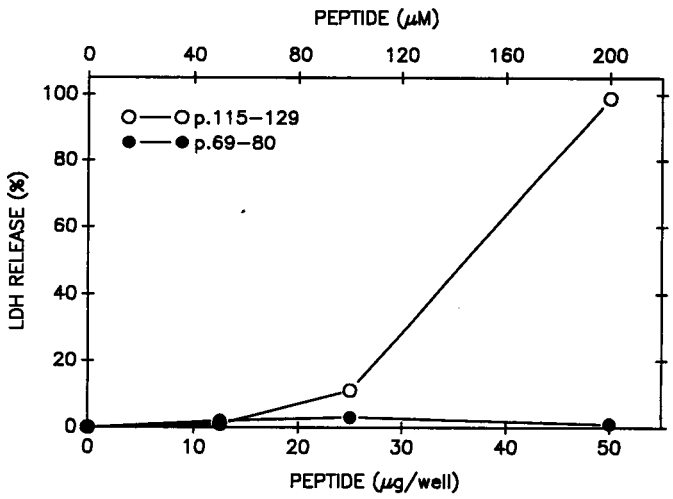

FIG. 6. **Peptide 115–129 reverses heparin neutralization of the cytolytic activity of myotoxin II.** Myotoxin II was mixed with heparin at a molar ratio of 1:1, and added to endothelial cells (10 μg toxin/well), resulting in a complete neutralization of its cytolytic activity. Increasing amounts of peptides 69–80 or 115–129, at the concentrations indicated, were added to the heparin:toxin mixtures, incubated for 15 min at room temperature, and then assayed for cytotoxicity, to determine if myotoxin II was relieved from neutralization by heparin. Peptides alone, at the same concentrations (indicated as μM in the *top axis* or as μg/well in the *bottom axis*), were devoid of cytotoxic effect (lactate dehydrogenase release or morphological changes). Each point represents mean ± S.D. of duplicates.

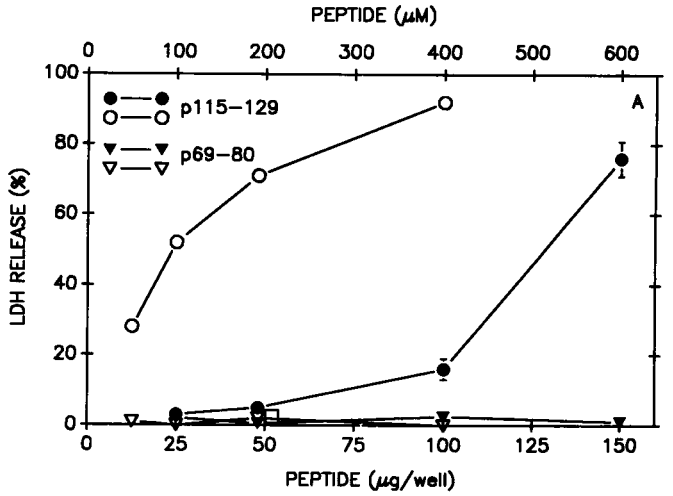

FIG. 7. **Cytolytic activity of peptide 115–129 on endothelial cells.** Cytotoxicity assay using peptides 69–80 and 115–129 dissolved in either cell culture medium (*filled symbols*), or PBS (*empty symbols*). Cytolysis was estimated by lactate dehydrogenase release, as described under "Experimental Procedures." Each point represents mean ± S.D. of duplicates. For comparison, addition of intact myotoxin II to endothelial cells results in 100% lactate dehydrogenase release at ~10 μg/well or ~5 μg/well, in culture medium or PBS, respectively. Peptide concentrations are indicated as μM (*top axis*) or as μg/well (*bottom axis*). The result obtained when peptide 115–129 was preincubated with heparin (2.4 μg peptide/μg heparin), in PBS, is indicated by the symbol (□).

than the binding of heparan sulfate, although the latter is less sulfated. This suggests some specificity for a heparin/heparan sulfate "backbone" in the interaction.

The minimal heparin oligosaccharide size capable of interacting with myotoxin II, in both direct binding and neutralization experiments, was the hexasaccharide. Similar results were recently described in the case of human secreted class II PLA_2 (37).

The formation of macromolecular complexes between heparin and myotoxin II, as shown by turbidimetry, is in agreement with previous observations of precipitated complexes in agarose gel (10), and implies a multivalent interaction involving at least two heparin-binding sites on this protein. Since myotoxin II can occur as a homodimer (5), at least one binding site might be available on each monomer.

Increasing the molar ratio of heparin:myotoxin II beyond 1:5 disrupted the macromolecular complexes, but did not reverse the neutralizing effect. The decreased retention of the [^{3}H]-heparin: myotoxin II complex on the nitrocellulose filters at high heparin proportions might be speculatively explained by a "masking" effect of the heparin chains, interfering with the binding of protein to the filter. For comparison, the same type of experiments (conducted with a fixed amount of protein and increasing amounts of labeled heparin) gave similar results as for myotoxin II in the case of platelet factor 4 (data not shown; multivalent interaction) (38), but showed classical saturation curves for antithrombin (39) and basic fibroblast growth factor (data not shown; both having monovalent heparin-binding sites).

MAb-3 has been characterized for its ability to neutralize myotoxin II *in vivo*, and in the cell model utilized in this report (10, 32). Our results suggest that MAb-3 and heparin recognize different sites on the toxin, and thus, that at least two molecular mechanisms for the neutralization of the cytolytic activity of myotoxin II exist.

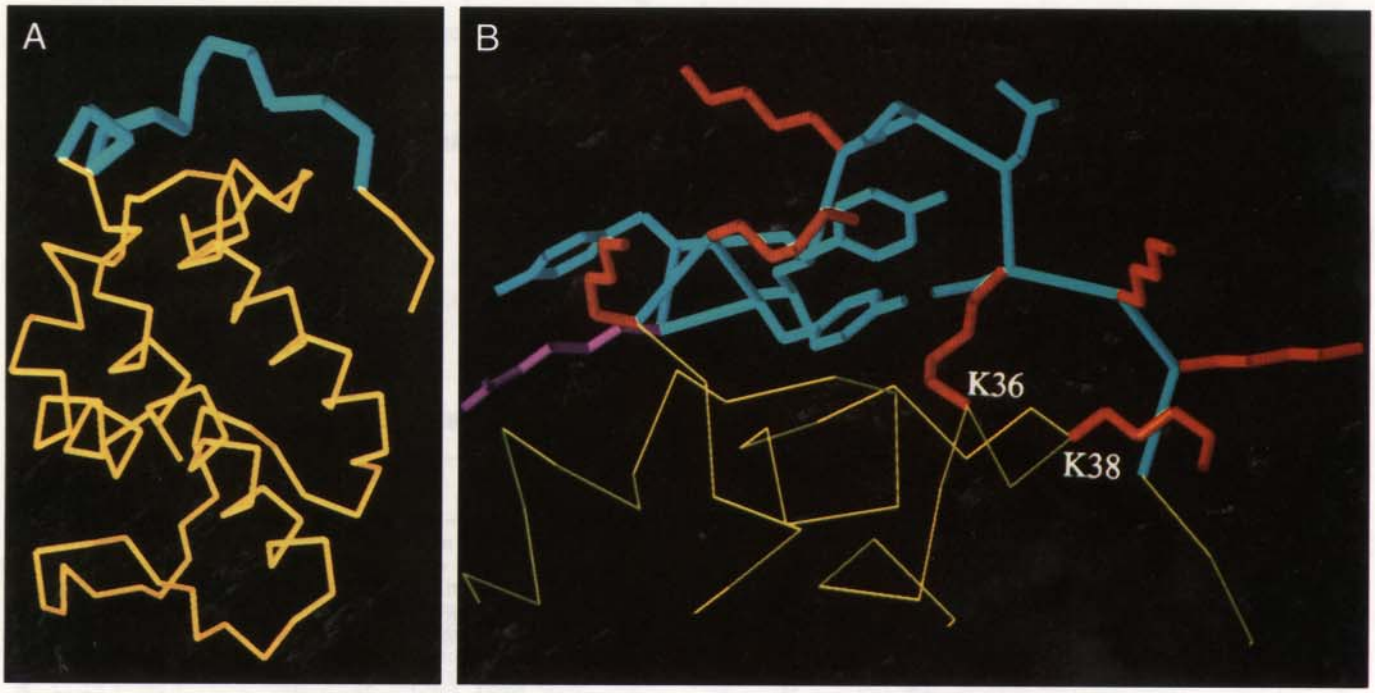

FIG. 8. **Molecular model of myotoxin II, based on the crystal structure of *A. p. piscivorus* K49 PLA_2.** *A*, myotoxin II α-carbon structure showing the region corresponding to residues 115–129 (*thick cylinders*) near the carboxyl terminus of the protein. *B*, the region corresponding to residues 115–129 is visualized with the side chains of the amino acids. Lysines are shown in red, and Arg-118 is shown in violet. Note the proximity of Lys-36 and Lys-38 to the region 115–129, enhancing its cationic character.

The epitope recognized by MAb-3 is discontinuous,[2] and has not been identified. On the other hand, the heparin-binding region(s) of myotoxin II was searched by the use of synthetic peptides. In a study of pancreatic PLA_2, evidence was obtained for a role of the 26 NH_2-terminal residues in its interaction with heparin (40). The corresponding synthetic peptide (residues 1–27) of myotoxin II was tested for heparin binding, since the overall architecture of PLA_2 molecules is highly conserved (28, 41, 42), but no evidence of interaction was obtained. Two synthetic peptides, 69–80 and 115–129, were selected for further analyses, on the basis of their high content of positively charged residues. Of these, only peptide 115–129 displayed heparin-binding ability, although with a decreased efficiency as compared to the whole protein. Interestingly, this peptide could reproduce the cytolytic effect of myotoxin II, at higher concentrations, while peptides 1–27 and 69–80 did not. In similarity with the action of myotoxins II (43) and III (44), the cytotoxic effect of peptide 115–129 was enhanced when the assay was performed in PBS, instead of medium. Altogether, the evidence strongly suggests that peptide 115–129 is part of a heparin-binding site of myotoxin II, which is also involved in cytotoxic action. Consequently, the neutralizing mechanism of heparin can be explained by its binding to a cationic region of myotoxin II that is critical for the cytolytic mechanism.

Based on the crystal structure of *A. p. piscivorus* K49 (34) and its high homology (75% sequence identity) (11) with myotoxin II, the three-dimensional structure of the latter was modeled, in order to locate peptide 115–129 and its context. The model showed that residues 115–129 are located at a highly exposed region, after the last α-helix or helix "E" (Fig. 8*A*). This region is probably not hindered by dimerization (assuming that myotoxin II dimerizes similar to other PLA_2s for which dimer crystal structures are known), and is clearly separated from the catalytic site (45, 46).

When peptide 115–129 was subjected to a dynamics simulation in solution, no significant changes in its conformation could be predicted. This suggests that its lower heparin-binding and cytolytic activities, in comparison to the whole protein, are probably not due to an altered conformation of the free peptide. Other possible explanations were investigated. First, the fact that myotoxin II can form dimers, and thus interact multivalently with heparin, would imply a higher avidity, as compared to the probable univalent interaction occurring with the small peptide. Second, visual inspection of the constructed model revealed that Lys-36 and Lys-38, although linearly distant from peptide 115–129, are conformationally very close to this segment (Fig. 8*B*). These two lysines might contribute to the higher level of binding observed for heparin, and especially for the modified heparins and the shortest oligosaccharides, in the case of the whole protein. In addition, Lys-36 and Lys-38 could also contribute to the cytolytic mechanism, since previous studies have shown that basic PLA_2 myotoxins require negative surface charges on liposomes in order to disrupt them by a mechanism that is independent of an enzymatic activity (47, 48). It is interesting to note that either Lys-38, or both Lys-36 and Lys-38, together with at least five positively charged residues in the region 115–129, are conserved in several myotoxic PLA_2s from Crotalid snakes (6, 49–52). In contrast, neurotoxic PLA_2 myotoxins from Elapid snake venoms do not exhibit these characteristics (53), suggesting possible differences in the molecular mechanisms of muscle damage between neurotoxic and non-neurotoxic PLA_2 myotoxins.

Based on sequence comparisons, it was predicted that the cytolytic region of myotoxic PLA_2s would be a cationic/hydrophobic sequence located at residues 79–87, just before helix E (53, 54). No similar prediction applies to myotoxin II. However, Lys-36 and Lys-38, together with the positively charged residues from peptide 115–129, are close to a group of three tyrosines (residues 117, 119, and 120; Fig. 8*B*), forming a cationic/hydrophobic combination in the three-dimensional

[2] B. Lomonte, unpublished data.

structure. Further work is necessary to define in detail the cytolytic site, its cellular target, and its mechanism of action.

Acknowledgments—We gratefully acknowledge Drs. A. Naggi and G. Grazioli (Istituto G. Ronzoni, Milano, Italy) for the preparation of 2-*O*- and 6-*O*-desulfated heparins, Dr. W. Murphy for the preparation of human aorta heparan sulfate, and Profs. Ulf Lindahl and Ingemar Björk for valuable advice.

REFERENCES

1. Rosenberg, P. (1990) in *Handbook of Toxinology* (Shier, W. T., and Mebs, D., eds) pp. 67–277, Marcel Dekker, Inc., New York
2. Rosenberg, P. (1986) in *Natural Toxins* (Harris, J. B., ed) pp. 129–174, Clarendon Press, Oxford
3. Gutiérrez, J. M., Lomonte, B., and Cerdas, L. (1986) *Toxicon* **24,** 885–894
4. Homsi-Brandeburgo, M. I., Queiroz, L. S., Santo-Neto, H., Rodrigues-Simioni, L., and Giglio, J. R. (1988) *Toxicon* **26,** 615–627
5. Lomonte, B., and Gutiérrez, J. M. (1989) *Toxicon* **27,** 725–733
6. Krizaj, I., Bieber, A. L., Ritonja, A., and Gubenöek, F. (1991) *Eur. J. Biochem.* **202,** 1165–1168
7. Kihara H., Uchikawa, R., Hattori, S., and Ohno, M. (1992) *Biochem. Int.* **28,** 895–903
8. Chwetzoff, S., Couderc, J., Frachon, P., and Menez, A. (1989) *FEBS Lett.* **248,** 1–4
9. Kjellén, L., and Lindahl, U. (1991) *Annu. Rev. Biochem.* **60,** 443–475
10. Lomonte, B., Tarkowski, A., Bagge, U., and Hanson, L. Å. (1994) *Biochem. Pharmacol.* **47,** 1509–1518
11. Francis, B., Gutiérrez, J. M., Lomonte, B., and Kaiser, I. I. (1991) *Arch. Biochem. Biophys.* **284,** 352–359
12. Lindahl, U., Cifonelli, J. A., Lindahl, B., and Rodén, L. (1965) *J. Biol. Chem.* **240,** 2817–2820
13. Höök, M., Riesenfeld, J., and Lindahl, U. (1982) *Anal. Biochem.* **119,** 236–245
14. Nagasawa, K., Inoue, Y., and Kamata, T. (1977) *Carbohydr. Res.* **58,** 47–55
15. Lloyd, A. G., Embery, G., and Fowler, L. J. (1971) *Biochem. Pharmacol.* **20,** 637–648
16. Danishefsky, I., and Steiner, H. (1965) *Biochim. Biophys. Acta* **101,** 37–45
17. Jaseja, M., Rej, R. N., Sauriol, F., and Perlin, A. S. (1989) *Can. J. Chem.* **67,** 1449–1456
18. Inoue, Y., and Nagasawa, K. (1976) *Carbohydr. Res.* **46,** 87–95
19. Iverius, P.-H. (1971) *Biochem. J.* **124,** 677–683
20. Lycke, E., Johansson, M., Svennerholm, B., and Lindahl, U. (1991) *J. Gen. Virol.* **72,** 1131–1137
21. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15,** 3932–3942
22. Pejler, G., Lindahl, U., Larm, O., Scholander, E., Sandgren, E., and Lundblad, A. (1988) *J. Biol. Chem.* **263,** 5197–5201
23. Lane, D. A., Denton, J., Flynn, A. M., Thunberg, L., and Lindahl, U. (1984) *Biochem. J.* **218,** 725–732
24. Bitter, T., and Muir, H. M. (1962) *Anal. Biochem.* **4,** 330–334
25. Laemmli, U. K. (1970) *Nature* **227,** 680–685
26. Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962) *Nature* **195,** 281–283
27. Valerio, R. M., Bray, A. M., Campbell, R. A., DiPasquale, A., Margellis, C., Rodda, S. J., Geysen, H. M., and Maeji, N. J. (1993) *Int. J. Pept. Protein Res.* **42,** 1–9
28. Renetseder, R., Brunie, S., Dijkstra, B. W., Drenth, J., and Sigler, P. B. (1985) *J. Biol. Chem.* **260,** 11627–11634
29. Maccarana, M., Casu, B., and Lindahl, U. (1993) *J. Biol. Chem.* **268,** 23898–23905
30. Bussolino, F., De Rossi, M., Sica, A., Colotta, F., Wang, J. M., Bocchietto, E., Padura, I. M., Bosia, A., Dejana, E., and Mantovani, A. (1991) *J. Immunol.* **147,** 2122–2129
31. Lomonte, B., and Kahan, L. (1988) *Toxicon* **26,** 675–689
32. Lomonte, B., Gutiérrez, J. M., Ramírez, M., and Díaz, C. (1992) *Toxicon* **30,** 239–245
33. Bolaños, R., and Cerdas, L. (1980) *Bol. Oficina Sanit. Panam.* **88,** 189–196
34. Holland, D. R., Clancy, L. L., Muchmore, S. W., Ryde, T. J., Einspahr, H. M., Finzel, B. C., Heinrikson, R. L., and Watenpaugh, K. D. (1990) *J. Biol. Chem.* **265,** 17649–17656
35. Lindahl, U., Thunberg, L., Bäckstrom, G., Riesenfeld, J., Nordling, K., and Björk, I. (1984) *J. Biol. Chem.* **259,** 12368–12376
36. Casu, B., Petitou, M., Provasoli, M., and Sina, Y. P. (1988) *Trends Biochem. Sci.* **13,** 221–225
37. Dua, R., and Cho, W. (1994) *Eur. J. Biochem.* **221,** 481–490
38. Ibel, K., Poland, G. A., Baldwin, J. P., Pepper, D. S., Luscombe, M., and Holbrook, J. J. (1986) *Biochim. Biophys. Acta* **870,** 58–63
39. Maccarana, M., and Lindahl, U. (1993) *Glycobiology* **3,** 271–277
40. Diccianni, M. B., Lilly, M., McLean, L. R., Balasubramaniam, A., and Harmony, J. A. K. (1991) *Biochemistry* **30,** 9090–9097
41. Westerlund, B., Nordlund, P., Uhlin, U., Eaker, D., and Eklund, H. (1992) *FEBS Lett.* **301,** 159–164
42. Scott, D. L., Achari, A., Vidal, J. C., and Sigler, P. B. (1992) *J. Biol. Chem.* **267,** 22645–22657
43. Lomonte, B., Tarkowski, A., and Hanson, L. Å. (1994) *Toxicon* **32,** 1359–1369
44. Bultrón, E., Thelestam, M., and Gutiérrez, J. M. (1993) *Biochim. Biophys. Acta* **1179,** 253–259
45. Dijkstra, B. W., Kalk, K. H., Hol, W. G., and Drenth, J. (1981) *J. Mol. Biol.* **147,** 97–123
46. Scott, D. L., White, S. P., Otwinowski, Z., Yuan, W., Gelb, M. H., and Sigler, P. B. (1990) *Science* **250,** 1541–1546
47. Díaz, C., Gutiérrez, J. M., Lomonte, B., and Gené, J. A. (1991) *Biochim. Biophys. Acta* **1070,** 455–460
48. Rufini, S., Cesaroni, P., Desideri, A., Farias, R., Gubensek, F., Gutiérrez, J. M., Luly, P., Massoud, R., Morero, R., and Pedersen, J. Z. (1992) *Biochemistry* **31,** 12424–12430
49. Kaiser, I. I., Gutiérrez, J. M., Plummer, D., Aird, S. D., and Odell, G. V. (1990) *Arch. Biochem. Biophys.* **278,** 319–325
50. Yoshizumi, K., Liu, S. Y., Miyata, T., Saita, S., Ohno, M., Iwanaga, S., and Kihara, H. (1990) *Toxicon* **28,** 43–54
51. Liu, S. Y., Yoshizumi, K., Oda, N., Ohno, M., Tokunaga, F., Iwanaga, S., and Kihara, H. (1990) *J. Biochem. (Tokyo)* **107,** 400–408
52. Maraganore, J. M., Merutka, G., Cho, W., Welches, W., Kézdy, F. J., and Heinrikson, R. L. (1984) *J. Biol. Chem.* **259,** 13839–13843
53. Kini, R. M., and Evans, H. J. (1989) *Int. J. Pept. Protein Res.* **34,** 277–286
54. Kini, R. M., and Iwanaga, S. (1986) *Toxicon* **24,** 895–905